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13. ABSTRACT (Maximum 200 Words)

Homologous recombination (HR) is an important tool for the repair of chromosomes damaged by various mutagenic chemicals including alkylating agents and DNA cross-linking agents, and by ionizing radiation that causes DNA double strand breaks. The dozen or so of genes and proteins that function in concert to mediate homologous recombination are collectively known as the *RAD52* epistasis group. In mammals, the breast tumor suppressors BRCA1 and BRCA2 regulate the efficiency of the HR machinery. Specifically, BRCA1 binds the Rad50/Mre11/Nbs1 complex and BRCA2 interacts with the Rad51 protein. The mechanism of the recombination reaction and the functional significance of the protein complexes containing the tumor suppressors and recombination proteins remain to be delineated. Our goal has been to establish an in vitro reaction with purified recombination factors and test the influence of BRCA2 protein on the efficiency of this reaction. As summarized in this final report, significant progress toward this goal has been made during the funding periods of this award.

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INTRODUCTION

In eukaryotes, genes of the *RAD52* epistasis group mediate *h*omologous recombination (HR) and the recombinational repair of DNA double-strand breaks (DSBs) (reviewed by Sung et al, 2000). Recent studies have implicated the HR machinery in the suppression of breast and other forms of tumors. Specifically, BRCA1 associates with and influences the activities of the protein complex consisting of three members of the *RAD52* group - Rad50, Mre11, and Nbs1 - required for the nucleolytic processing of DSBs. Consistent with this observation, BRCA1 is necessary for normal levels of homologous recombination and resistance to genotoxic agents. Rad51, another key member of the *RAD52* group, binds to the BRC repeats and the carboxyl-terminal domain of BRCA2. Mouse embryos harboring a Brca2 truncation are hypersensitive to ionizing radiation. In addition, BRCA2 deficient cell lines are impaired for the ability to assemble Rad51 nuclear foci upon DNA damaging treatment and deficient in HR. Many of the *RAD52* group genes have also been found mutated in primary tumors (reviewed by Pierce et al, 2001). Using a biochemical approach, we have been conducting studies directed at reconstituting the HR machinery and deciphering the molecular functions of BRCA1 and BRCA2 in the HR reaction.

BODY

In DSB repair mediated by HR, a ssDNA intermediate is utilized by the recombination machinery to invade the sister chromatid or homologous chromosome to form a DNA joint molecule called D-loop. D-loop formation provides a primer template junction to initiate DNA synthesis. Subsequent steps involve the resolution of recombination DNA intermediates and DNA ligation. The DNA strand invasion step, also known as the homologous DNA pairing and strand exchange reaction, is catalyzed by the Rad51 protein, the structural and functional homolog of the *E. coli* general recombinase enzyme RecA (Sung, 1994; Sung et al, 2000). Below we summarize our studies on Rad51 and its associated factors.

Basis for Avid Homologous DNA Pairing and Strand Exchange by Human Rad51 (Sigurdsson et al, 2001a, J. Biol. Chem. 276: 8798-8806)

Among members of the *RAD52* group, the *RAD51* encoded product is of particular interest because of its structural and functional similarities to the *E. coli* recombination protein RecA. RecA promotes the pairing and strand exchange between homologous DNA molecules to form heteroduplex DNA, an enzymatic activity believed to be germane for the central role of RecA in recombination and DNA repair processes. Likewise, homologous DNA pairing and strand exchange activities have been shown for *S. cerevisiae* Rad51 (yRad51; Sung, 1994; Sung et al, 2000). Under optimized conditions, the length of heteroduplex DNA joints formed by yRad51 and RecA can extend over quite a few kilo base pairs. In published studies, human Rad51 (hRad51) was found to have the ability to make DNA joints but the maximal potential for forming only about one kilo base pairs of heteroduplex DNA (Baumann et al, 1996; Gupta et al, 1997). Furthermore, while yRad51 and RecA require their cognate single-strand DNA binding factors, SSB and rap, for optimal recombinase activity, hRPA has been suggested to stimulate the hRad51-mediated homologous pairing and strand exchange reaction only when the hRad51 concentration is sub-optimal (Baumann et al, 1999).

Given the central role of hRad51 in recombination processes and the fact that the activities of hRad51 are apparently subject to modulation by tumor suppressor proteins such as

BRCA2, establishing an efficient hRad51-mediated DNA strand exchange system will be important for dissecting the functional interactions among hRad51, other recombination factors, and tumor suppressors. We have explored a variety of reaction parameters that could influence the recombinase activity of hRad51. We demonstrated that under certain conditions, hRad51 makes DNA joints avidly and promotes highly efficient strand exchange over at least 5.4 kilo base pairs. Importantly, under the new reaction conditions, the efficiency of the hRad51-mediated DNA strand exchange reaction is strongly dependent on hRPA over a wide range of Rad51 concentrations tested. We provided compelling evidence to indicate that the relatively high levels of divalent anions present in our reaction system stimulate homologous DNA strand exchange by modulating the affinity of the hRad51-ssDNA complex for the incoming duplex molecule. These results have been reported in Sigurdsson et al (2001a).

Role of ATP Binding and Hydrolysis in Rad51 Functions

Unlike its bacterial counterpart RecA and its yeast counterpart yRad51, which both require ATP for loading onto DNA, human Rad51 is thought to bind DNA in an ATP-independent manner. Since ATP is clearly required for homologous DNA pairing and strand exchange by human Rad51 (Baumann et al, 1996; Gupta et al, 1997; Sigurdsson et al, 2001a), we wished to define the role of ATP in DNA binding and DNA strand exchange. To this end, we constructed two mutant variants of hRad51, changing the conserved Walker lysine residue to either alanine (K133A) or arginine (K133R). Based on extensive studies done with the equivalent Walker mutants of other ATPases, we expected that the former mutant (K133A) would not bind ATP, whereas the latter (K133R) would retain the ability to bind ATP, with both being defective in ATP hydrolysis. We have expressed these two Walker Rad51 mutants in E. coli and purified them to near homogeneity. We have examined the homologous DNA pairing activity of the two Rad51 mutants. While Rad51 and Rad51K133R catalyzed pairing between the DNA substrates, Rad51K133A was inactive. Interestingly, the Rad51 K133R mutant is more apt at pairing the substrates than the wild type protein, and with the wild type Rad51 protein, the level of homologous pairing is higher with the non-hydrolyzable ATP analogue AMP-PNP than ATP. These results are consistent with the premise that ATP hydrolysis mediates turnover of Rad51 from the ssDNA template, and therefore a more stable Rad51-ssDNA nucleoprotein filament is formed when the bound nucleotide is not hydrolyzed by Rad51, leading to a higher level of homologous pairing. Importantly, our biochemical results are highly congruent with published genetic studies on the aforementioned *rad51* alleles (Morrison et al, 1999; Stark et al, 2002). We are currently preparing a manuscript on these findings to submit to a peer-reviewed journal.

Recombination Mediator Function in the Rad51B-Rad51C Complex. (Sigurdsson et al, 2001b, Genes & Develop. 15:3308-3318)

hRad51 protein mediates the homologous DNA pairing and strand exchange reaction that is central to all recombination processes. Our studies have shown that the heterotrimeric ssDNA binding factor hRPA is an important accessory factor for Rad51-mediated homologous DNA pairing and strand exchange. Interestingly, hRPA can also compete with hRad51 for binding sites on the ssDNA template and markedly suppress pairing and strand exchange efficiency. We have demonstrated that the stoichiometric complex of the human Rad51B and Rad51C proteins, homologs of the *Saccharomyces cerevisiae* Rad55 and Rad57 proteins, can help overcome the suppressive effect of hRPA on hRad51-catalyzed DNA pairing and strand exchange, thus identifying the Rad51B-Rad51C complex as a mediator of

recombination. Our results have also shown that Rad51B-Rad51C complex has ssDNA binding and ssDNA-stimulated ATPase activities.

Like other members of the RecA/Rad51 class of recombinases, hRad51 assembles onto ssDNA to form a nucleoprotein filament in an ATP-dependent manner. Extensive biochemical studies with RecA have indicated that the search for DNA homology, DNA joint formation, and DNA strand exchange all occur within the confines of the RecA-ssDNA nucleoprotein filament. The assembly of the recombinase-ssDNA nucleoprotein filament is therefore the critical first step in the homologous DNA pairing and strand exchange reaction. The Rad51B-Rad51C complex is likely required for the assembly of the Rad51-ssDNA nucleoprotein filament *in vivo*. These results are documented in Sigurdsson et al (2001b).

hRad54 Supercoils DNA and Promotes Rad51-mediated D-loop Formation (Sigurdsson et al, 2002, J. Biol. Chem. 277:42790-42794)

hRad54, a member of the Swi2/Snf2 family of proteins, has been overexpressed in insect cells by the use of a recombinant baculovirus and purified to near homogeneity. Rad54 hydrolyzes ATP in the presence of ϕX174 duplex DNA or the (+) strand, with the former being the more effective co-factor. No ATPase activity was observed when DNA is omitted. We have found that hRad54, at the expense of ATP hydrolysis, generates unconstrained, compensatory negative and positive supercoils in duplex DNA. Our results, (Sigurdsson et al, 2002; Van Komen et al, 2000) together with those published by others (Ristic et al, 2002), indicate that the free energy from ATP hydrolysis is used to fuel translocation of hRad54 on duplex DNA. Furthermore, the negative supercoils that accumulate lead to transient DNA strand opening, as indicated by a marked sensitivity of the DNA to the single-strand specific nuclease P1. Purified hRad54 protein also binds avidly to hRad51 immobilized on Affi-gel beads in the absence of DNA, indicating direct interaction between the two. It is interesting to note that complex formation between Rad51 and Rad54 occurs in the absence of ATP. In the D-loop reaction, hRad54 has been found to greatly accelerate the rate of homologous pairing by hRad51. These results have been published (Sigurdsson et al, 2002).

Role of Rad54B in DNA Joint Formation

Like Rad54, Rad54B protein possesses the seven Swi2/Snf2 signature motifs. Deletion of Rad54B in human cells compromises HR-mediated gene targeting. Rad54B exhibits an expression pattern similar to that of other *RAD52* epistasis group members and it colocalizes in the nucleus with other HR proteins, including Rad54 and the tumor suppressor BRCA1. We have expressed Rad54B protein in insect cells and have devised a chromatographic scheme to purify it to near homogeneity. Utilizing our published system for examining the DNA supercoiling activity of Rad54 (Sigurdsson et al, 2002), we have found that Rad54B also generates supercoils in DNA. This reaction is dependent on ATP hydrolysis by Rad54B, as revealed by substituting ATP with the non-hydrolyzable analogues. We have tested whether DNA supercoiling by Rad54B induces DNA strand opening by utilizing an assay that measures the sensitivity of DNA to the single-strand specific nuclease P1. The results indicated that the negative supercoiling induced by Rad54B indeed causes transient strand opening in the duplex DNA. This strand opening activity of Rad54B is again dependent upon ATP hydrolysis, as sensitivity to P1 nuclease was not seen when ATP was substituted with non-hydrolyzable analogues. Since Rad51 and Rad54B interact physically and, we wished to examine Rad54B for the ability to promote Rad51-mediated homologous pairing. In these experiments, robust D-loop formation was seen.

The control experiments showed that neither Rad51 nor Rad54B alone was capable of forming any D-loop, thus confirming a functional cooperation of these two proteins in the D-loop reaction. We are in the process of preparing a manuscript to document these findings to submit to a peer-reviewed journal.

BRCA2 protein and the Rad51 associated complex

We have expanded considerable effort to express full-length human BRCA2 protein in the baculovirus system and have thus far obtained only a small amount of soluble protein. We have recently achieved expression of the BRCA2 in yeast cells and are in the process of devising a protocol for its purification. We have generated a library of Rad51 mutants by its passage through a mutagenic bacterial strain. We are in the process of screening this mutagenized plasmid collection using the negative two-hybrid strategy to find variants of Rad51 that are defective in binding BRCA2. These Rad51 mutants will be studied genetically for their ability to promote HR and DSB repair by HR, so as to ascertain the significance of the Rad51/BRCA2 protein complex.

KEY RESEARCH ACCOMPLISHMENTS

- Purification of hRad51 and hRPA and establishing biochemical systems for studying the functional interactions between these two factors in HR reactions.
- Providing mechanistic information on the role of ATP binding and hydrolysis in Rad51-mediated HR reactions.
- Expression and purification of human Rad51B-Rad51C complex and extensive characterization of its DNA binding and ATPase activities and also its functional interactions with human Rad51 in the homologous DNA pairing reaction.
- Cloning, expression and purification of human Rad54 protein and extensive characterization of its ATPase and DNA supercoiling activities and also its functional interactions with human Rad51 in DNA supercoiling and the homologous DNA pairing reaction.
- Cloning, expression and purification of human Rad54B protein and extensive characterization of its ATPase and DNA supercoiling activities and also its functional interactions with human Rad51 in DNA supercoiling and the homologous DNA pairing reaction.
- Generation of Rad51 mutants defective in specific interactions with other HR factors.

LIST OF PERSONNEL

Patrick Sung, Ph.D.

Stefan Sigurdsson, Ph.D.

Stephen Van Komen, Ph. D.

REPORTABLE OUTCOMES

(1) Publications *(copies enclosed)*:

Sung, P., Trujillo, K., and Van Komen, S. 2000. Recombination factors of *Saccharomyces cerevisiae*. Mutat. Res. 451: 257-275.

Sigurdsson, S., Trujillo, K., Song, B-W., Stratton, S., and Sung, P. 2001a. Basis for avid homologous DNA strand exchange by human Rad51 and RPA. J. Biol. Chem. 276: 8798-8806.

Sigurdsson, S., Van Komen, S., Bussen, W., Schild., D., Albala, J., and Sung, P. 2001b. Mediator function of the Rad51B-Rad51C complex in Rad51/RPA –mediated DNA strand exchange. *Genes & Develop.* 15:3308-3318.

Sigurdsson, S., Guo, X.Z., Van Komen, S., Petukhova, G., and Sung, P. 2002. Homologous DNA pairing by human Rad51 and Rad54 proteins. J. Biol. Chem. 277: 42790-42794.

(2) Meeting Abstracts: NONE

(3) Presentations *(selected from over forty since 1999)*:

1999 Invited Speaker, Keystone Symposium on Molecular Mechanisms in DNA Replication and Recombination, Taos, New Mexico. Title of Presentation: "Mechanism of Heteroduplex DNA Formation".

1999 Invited Speaker, FASEB Conference on the Mechanism of Genetic Recombination, Snowmass, Colorado. Title of Presentation: "Synergistic Interactions of Rad Proteins in Recombination & Repair."

2000 Invited Speaker, National Cancer Institute - Frederick Cancer Research and Development Center. Title of Presentation: "Role of Rad52 Group Recombination Proteins in Heteroduplex DNA Formation".

1999 Invited Speaker, Department of Biochemistry, University of North Carolina at Chapel Hill. Title of Presentation: "DNA Strand Exchange Reactions in Homologous Recombination".

2000 Invited Speaker, Colloquium on Links Between Recombination and Replication: Vital Roles of Recombination, Irvine, California. Title of Presentation: "Roles of *RAD52* Group Genes and Proteins in Recombination and Repair". Organized by the U.S. National Academy of Sciences.

2000 Invited Speaker, EMBO Workshop on Mechanisms of Genetic Recombination, Seillac, France. Title of Presentation: "Rad51 and Rdh54 Constitute a DNA Supercoiling Motor Indispensable for D-loop Formation".

2000 Invited Speaker, National Cancer Institute - Frederick Cancer Research and Development Center. Title of Presentation: "Role of Rad52 Group Recombination Proteins in Heteroduplex DNA Formation".

2001 Invited Speaker, Gordon Research Conference on Mammalian DNA Repair, Ventura, California. Title of Presentation: "Mechanisms of Rad52 Group Recombination Factors".

2001 Invited Speaker, Gordon Research Conference on Radiation Oncology, Ventura, California. Title of Presentation: "A Hierarchy of Functional & Physical Interactions Among the RAD52 Group Recombination Factors".

2001 Invited Speaker, FASEB Summer Research Conference on Helicases: Structure, Function and Roles in Human Disease, Saxtons River, Vermont. Title of Presentation: "Functional Interactions Among RAD52 Group Proteins in Homologous Recombination".

2001 Invited Speaker, FASEB Research Conference on Recombination Mechanisms. Snowmass Colorado. Title of Presentation: "Basis for Avid Homologous DNA Strand Exchange by Human Rad51 Recombinase and RPA".

2001 Invited Speaker, Workshop on DNA Repair and Recombination, organized by the National Institutes of Health. Title of Presentation: "An Overview of Homologous Recombination Mechanism in Eukaryotic Cells".

2001 Invited Speaker, Columbia University. Title of Presentation: "DNA Strand Exchange by Human Rad51 and RPA".

2001 Invited Speaker, BRCA1 and Breast Cancers. Title of Presentation: "Functional Interactions that Govern the Integrity of the Homologous Recombination Machinery". Organized by the National Cancer Institute.

2002 Invited Speaker, Keystone Conference on Mechanism of DNA Replication and Recombination. Title of Presentation: "Recombination Factors that Influence the Rad51 Recombinase Activity".

2002 Invited Speaker & Session Chair, EMBO Workshop on Mechanisms of Genetic Recombination. Chaired session on "Recombination Proteins". Title of Presentation: "Action Mechanism of Yeast and Human Rad54".

2002 Invited Speaker, Gordon Conference on Mutagenesis. Bates College, Maine. Title of Presentation: "Crosstalk Among Rad51, Rad54, and RPA in DNA Joint Formation".

2002 Invited Speaker, Yale University. Title of Presentation: "DNA Double-strand Break Repair in Eukaryotes".

2002 Invited Speaker, Cornell University. Title of Presentation: "RAD52 Group of Recombination Factors".

2002 Invited Speaker, Meeting on DNA Recombination and Repair, Cold Spring Harbor Laboratory. Title of Presentation: "Mechanism of Heteroduplex Formation".

2003 Invited Speaker and Discussion Leader: Gordon Research Conference on Mammalian DNA Repair. Ventura, California. Title of Presentation: "Homologous Recombination: Mediators and Regulators".

2003 Invited Speaker: Stanford University Tumor Biology Program. Title of Presentation: "Mechanism of Homology-directed Repair of DNA Strand Breaks".

2003 Invited Speaker: Memorial Sloan-Kettering Cancer Center. Title of Presentation: "Mechanisms of the RAD52 Group Recombination Proteins".

2003 Invited Speaker, ASBMB meeting: Title of Presentation: "DNA Double-Strand Break Repair by Homologous Recombination".

2003 Invited Speaker: University of North Carolina at Chapel Hill. Title of Presentation: "Hierarchy of Functional Interactions that Govern the Efficacy of Homologous Recombination".

CONCLUSIONS

Our molecular studies on the human recombination factors shed light on how these factors functionally cooperate in mediating the homologous DNA pairing and strand exchange reaction central to all recombination processes. The results have led to better understanding of the human recombination machinery and the role of this machinery in tumor suppression. The biochemical systems derived from our work should prove to be a useful tool for deciphering the role of breast tumor suppressors in the recombination reaction and rationalizing the function of the recombination machinery in tumor suppression.

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Ristic, D., Wyman, C., Paulusma, C., and Kanaar, R. 2001. The architecture of the human Rad54-DNA complex provides evidence for protein translocation along DNA. Proc. Natl. Acad. Sci. U.S.A. 84: 8454-8460.

Sigurdsson, S., Trujillo, K., Song, B-W., Stratton, S., and Sung P. 2001a. Basis for avid homologous DNA strand exchange by human Rad51 and RPA. J. Biol. Chem. 276: 8798-8806.

Sigurdsson, S., S. Van Komen, W. Bussen, D. Schild., J. Albala, and Sung, P. 2001b. Mediator function of the Rad51B-Rad51C complex in Rad51/RPA –mediated DNA strand exchange. Genes & Develop. 15:3308-3318.

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Stark, J.M., Hu, P., Pierce, A.J., Moynahan, M.E., Ellis, N., and Jasin, M. 2002. ATP hydrolysis by mammalian RAD51 has a key role during homology-directed DNA repair. J. Biol. Chem. 277:20185-201å94.

Sung, P. 1994. Catalysis of ATP dependent homologous DNA pairing and strand exchange by the yeast RAD51 protein. Science 265:1241-1243.

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Van Komen, S., Petukhova, G., Sigurdsson, S., Stratton, S., and Sung, P. 2000. Superhelicity-driven homologous DNA pairing by yeast recombination factors Rad51 and Rad54. Mol. Cell 6: 563-572.

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Review

Recombination factors of *Saccharomyces cerevisiae*

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Abstract

The budding yeast *Saccharomyces cerevisiae* has been an excellent genetic and biochemical model for our understanding of homologous recombination. Central to the process of homologous recombination are the products of the *RAD52* epistasis group of genes, whose functions we now know include the nucleolytic processing of DNA double-stand breaks, the ability to conduct a DNA homology search, and the capacity to promote the exchange of genetic information between homologous regions on recombining chromosomes. It is also clear that the basic functions of the *RAD52* group of genes have been highly conserved among eukaryotes. Disruption of this important process causes genomic instability, which can result in a number of unsavory consequences, including tumorigenesis and cell death.

1. Prologue

In addition to creating genetic diversity, homologous recombination is also an important tool for repairing DNA double-strand breaks (DSBs). Furthermore, meiotic recombination helps establish stable interactions between chromosomal homologs, and as such, is indispensable for the proper disjunction of chromosomes in the first meiotic division (reviewed in Refs. [47,71,82]).

Much of our understanding of homologous recombination processes in eukaryotes has originated from studies conducted in the budding yeast *Saccharomyces cerevisiae*. The common denominator of many recombination processes in *S. cerevisiae* (henceforth referred to as yeast) is a DNA DSB, formed either as a result of exposure of cells to break-inducing radiation and chemicals or as part of developmental programs. The most notable examples of the latter class of recombination processes are mating type switching, being initiated by a site-specific DSB made by the HO endonuclease, and meiotic recombination, which occurs by way of DSBs introduced by a complex of proteins including Spo11 as the catalytic subunit. Once a DSB is formed, the ends of the break are subjected to processing by exonucleolytic activities and the single-stranded DNA tails thus formed will be channeled into one of a number of homologous recombination pathways as discussed below and elsewhere [66]. Alternatively, the DNA ends can simply be rejoined via the non-homologous DNA end-joining pathway, with or without further processing. A synopsis of the major homologous recombination pathways and their genetic and biochemical requirements is given below. Comprehensive reviews on non-homologous DNA

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end-joining have been published recently [32,107], and this topic will not be dealt with here.

2. Recombination processes

2.1. Classical recombination

In classical recombination, the DSB is processed by an exonuclease activity, digesting away a substantial portion of the DNA strands that contain the 5′ ends of the break. This end-processing reaction results in long (as long as 1 kb or more) ssDNA tails that have a 3′ extremity. These ssDNA tails are utilized for the nucleation of a number of recombination factors to yield a nucleoprotein complex that has the ability to conduct a DNA homology search to locate an intact DNA homolog, which could be either the sister chromatid or the homologous chromosome. Invasion of the homolog in a reaction called "homologous DNA pairing and strand exchange" yields a joint between the recombining molecules (Fig. 1). When the recombining DNA molecules encompass different alleles of the same gene, then DNA mismatches will form in the DNA joint, giving rise to heteroduplex DNA. Correction of the DNA mismatches results in the conversion of one of the recombining alleles to the other. Heteroduplex DNA formation followed by DNA mismatch correction represents an important means for gene conversion in yeast, although it is not the only route. In considering this well recognized DNA DSB repair model for homologous recombination (variants of this model have been discussed by Paques and Haber [66]), one important point to bear in mind is that the 3′ ssDNA tails arising through DSB end-processing, rather than the DSB per se, are in fact the substrate utilized by the recombination machinery for mediating subsequent reactions.

While meiotic recombination mainly involves chromosomal homologs, it is believed that most of the recombination events during mitotic growth occur in the late S and G2 phases and involve the sister chromatids. However, in yeast, there is considerable capacity to carry out allelic (interchromosomal) recombination during mitotic growth, and allelic recombination appears to have a somewhat different genetic requirement than sister chromatid-based recombination ([66]; see later). Mutations in genes

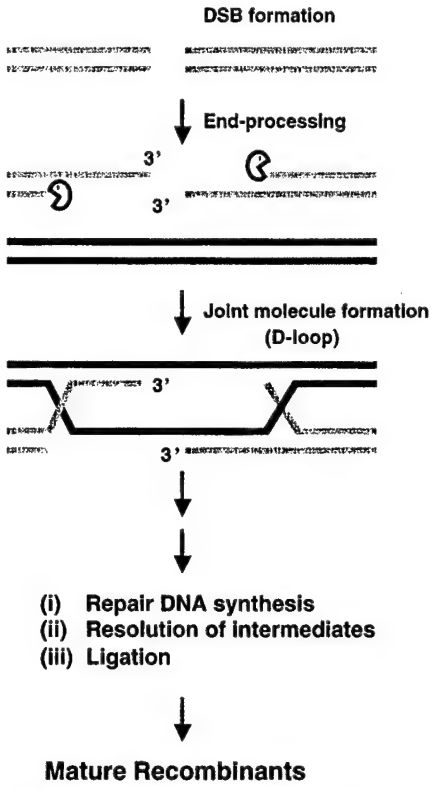

Fig. 1. Recombination induced by DNA DSBs. The ends of DNA DSBs are processed nucleolytically to yield long 3′ single-strand tails. Nucleation of recombination factors onto the single-strand tails leads to a search for a DNA homolog and pairing with the homolog to form a joint molecule called D-loop. Concurrent and subsequent steps include DNA synthesis to replace the genetic information eliminated during end-processing, resolution of the Holliday intermediates, and DNA ligation to complete the recombination process.

(e.g., *RAD50*, *MRE11*, and *XRS2*) that are believed to mediate sister chromatid-based recombination could in fact lead to higher levels of recombination between homologs. It has been suggested that when sister chromatid-based recombination is inactivated, the recombinogenic DNA substrates are channeled more often into interchromosomal recombination pathways.

2.1.1. The players in classical homologous recombination: RAD52 epistasis group

Genetic screens based mainly on sensitivity to ionizing radiation have identified a large number of genetic loci required for the repair of DNA breaks,

and many of these genes have subsequently been shown to be needed for efficient mating type switching, mitotic recombination, and meiotic recombination. These genes — *RAD50*, *RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, *RDH54/TID1*, *MRE11*, and *XRS2* — are collectively known as the *RAD52* epistasis group [6,29,48,66,71,90]. Below, we provide a synopsis of the biochemistry of the products of these genes and their likely roles in enzymatic reactions that lead to the formation of recombinants.

2.1.1.1. Biochemistry of homologous recombination DNA end-processing

Role of Rad50, Mre11, and Xrs2 in end-processing. A good body of evidence has linked *RAD50*, *MRE11*, and *XRS2* to DSB end-processing during recombination processes [66]. Rad50 is a highly conserved member of the Structural Maintenance of Chromosomes (SMC) family of proteins, which functions in different aspects of chromosomal metabolism (reviewed in Ref. [35]). Mre11 is also a highly conserved protein and shows homology to phosphodiesterases. Rad50 and Mre11 are the respective homologs of *Escherichia coli* SbcC and SbcD [84], which combine to form a complex that has ssDNA endonuclease and ATP-dependent exonuclease activities, and also an ability to open DNA hairpins; SbcD is the catalytic subunit of this nuclease complex [20]. Consistent with these observations, Mre11 from both yeast [28,60,108] and humans [69,106] exhibits ssDNA endonuclease activity, a 3′ to 5′ exonuclease activity, and hMre11 also possesses an ability to cleave DNA hairpins [69,70]; whether yMre11 acts on DNA hairpins remains to be determined. hMre11 combines with hRad50, and the resulting complex has enhanced exonuclease activity [69]. The exonuclease activity of hRad50–hMre11 complex is not stimulated by ATP, thus marking a major difference between this human complex and the bacterial SbcC–SbcD complex. Mre11 from both yeast and humans, like its bacterial counterpart SbcD, specifically requires manganese for the activation of its nuclease activity.

The human Rad50–Mre11 complex combines, primarily or exclusively through Mre11 [70], with a third protein called p95 [22]. Recently, p95 was found to be mutated in the cancer prone disease Nijmegen breakage syndrome (NBS), and is now also called NBS1 or nibrin [16,110]. NBS1/nibrin modulates the nuclease function of the Rad50–Mre11 complex, making it possible for the complex containing the protein trio to act efficiently on different types of DNA hairpins and also endonucleolytically and in an ATP-dependent manner, on 3′ ssDNA tails that border a duplex region. The Rad50–Mre11–NBS1 complex has a modest ability to unwind duplex DNA, resulting in DNA strand separation. DNA unwinding is stimulated by ATP, and mutating the nucleotide binding fold in Rad50 renders the mutant Rad50 containing protein complex insensitive to ATP in DNA unwinding and nuclease functions [70]. NBS cells, like ataxia telangiectasia (AT) cells, appear to be defective in different cell cycle checkpoints. Based on this phenotype of NBS cells, it has been proposed that NBS1 relays the detection of DNA lesions to the cell cycle checkpoint machinery (reviewed in Ref. [88]). However, the results of Paull and Gellert [70] have clearly demonstrated that NBS1 is in fact also important for the expression of the full repertoire of biochemical activities of the Mre11-associated complex.

In yeast, Xrs2 combines with the Rad50–Mre11 complex through the Mre11 subunit [43,108]. Although Xrs2 is considered the yeast equivalent of NBS1, Xrs2 is only distantly related to NBS1 in amino acid sequence, with homology noted only in the amino-termini of the two factors where putative protein–protein interaction domains are located (reviewed in Ref. [27]). There is no information available as to the biochemical functions of Xrs2 and its role in modulating the activities of Rad50 and Mre11.

Possible means for processing DNA ends. Studies in yeast have clearly indicated that the ends of DNA DSBs are processed to yield 3′ ssDNA tails (Fig. 1), predicting a 5′ to 3′ exonuclease activity in the end-processing reaction. It was therefore rather surprising to find that hMre11 and yMre11 both have a 3′ to 5′ exonuclease activity but are apparently devoid of a significant 5′ to 3′ exonuclease activity [28,60,69,106,108]. To reconcile this paradox, it has been suggested that the Mre11-associated complex functionally cooperates with a DNA helicase to unwind DNA from the ends, creating an open structure for the endonucleolytic function of Mre11 to act on (Fig. 2). Since a short single-stranded region may be

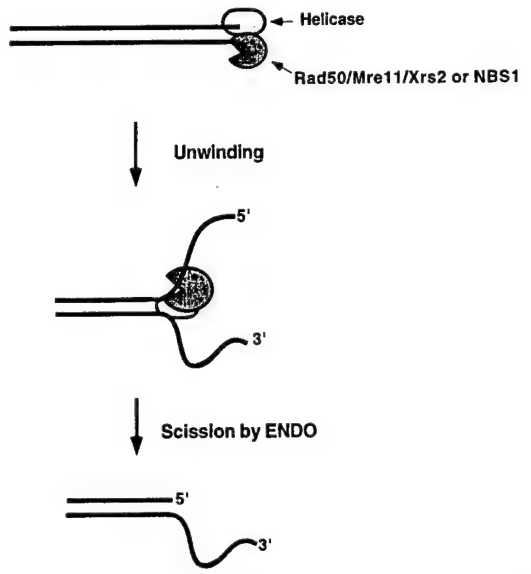

Fig. 2. Possible mechanism for DSB end-processing. It is postulated that a higher order complex of a DNA helicase and the trio of Rad50, Mre11, and Xrs2 is recruited to the DNA ends. DNA strand separation by the helicase then creates a splayed DNA structure, the 5′ overhang of which is then excised by the endonucleolytic activity of Mre11 to generate the 3′ ssDNA tail observed in genetic experiments.

required for the loading of a DNA helicase and activation of DNA unwinding, it seems possible that the 3′ to 5′ exonuclease activity of Mre11 may, at least under some circumstances, be involved in generating a short 5′ ssDNA overhang for the loading and activation of the DNA helicase.

Genetic results have clearly indicated that Mre11 is not the only nuclease capable of processing DNA ends during mitotic recombination, and that the Mre11 complex must play another role in recombination [66]. Since the sister chromatid appears to be the preferred DNA homolog used in directing recombinational repair, one plausible idea is that Mre11 together with Rad50 and Xrs2 mediate the type of sister–sister chromatid interactions indispensable for the efficient repair of lesions by sister chromatid-based recombination ([66]; John Petrini, personal communication).

Unlike the situation in mitotic cells, the processing of DNA DSBs during meiotic recombination is largely or completely dependent on the Mre11-associated nuclease activity. As discussed elsewhere [10,49], a putative topoiosmerase activity in Spo11 is likely to be responsible for the generation of meiosis-specific DSBs, and Spo11 has been shown to remain covalently attached to the 5′ termini of the DNA breaks [49]. Thus, it appears that only the Mre11-associated protein complex is capable of removing Spo11 covalently attached to the 5′ termini of the meiosis-specific DSBs.

Multifunctional nature of Rad50, Mre11, and Xrs2. Genetic analyses have revealed that Rad50, Mre11, and Xrs2 work in conjunction with Spo11 to introduce meiosis specific DNA DSBs. Truly remarkable are the observations that the trio of Rad50, Mre11, and Xrs2 are also required for the maintenance of telomere length and for non-homologous DNA end joining. Notably, the Mre11 nuclease activity appears to be dispensable for its functions in meiotic DSB formation, telomere maintenance, and DNA end-joining. The readers can find more information on these topics in recent reviews [32,66,107].

Heteroduplex DNA formation

Roles of Rad51, Rad52, Rad54, Rad55, Rad57, Rdh54 / Tid1, and RPA in heteroduplex DNA formation. Genetic and biochemical studies have revealed a role for Rad51, Rad52, Rad54, Rad55, Rad57, Rdh54, and RPA in the utilization of recombinogenic ssDNA substrates for the formation of heteroduplex DNA (reviewed in Ref. [66]). Here, we will describe in some detail the homologous DNA pairing and strand exchange reaction that is responsible for the generation of heteroduplex DNA, and will summarize the current state of knowledge of the biochemical functions of these recombination factors. The properties and salient features of the various recombination factors are also given in Table 1.

Rad51 recombinase. The *RAD51* encoded product is homologous to the *E. coli* general recombinase RecA [2,9,89], most notably in the regions of the latter that are concerned with catalytic functions, including the motifs involved in DNA binding and in nucleotide binding and hydrolysis [11,81]. Genetic studies have clearly implicated *RAD51* in recombination processes, and *rad51* mutants exhibit the type of phenotypes expected for eukaryotic homolog of RecA [2,9,89]. Direct evidence supporting the notion that Rad51 is a true functional RecA homolog has come from biochemical studies which demonstrated that (i) in the presence of ATP, Rad51 assem-

Table 1
The recombination factors of yeast

Protein	Size	Biochemical function	*E. coli* homolog	Human homolog	Notable features
Rad50	152,545	DNA-binding	SbcC	hRad50	Member of SMC family; forms complex with Mre11 and Xrs2
Mre11	77,630	ssDNA endonuclease 3′ to 5′ exonuclease	SbcD	hMre11	Homology to phosphodiesterases; forms complex with Rad50 and Xrs2
Xrs2	96,366	not known	none	NBS1	Forms complex with Rad50 and Mre11
Rad51	42,943	ATP-dependent homologous DNA pairing and strand exchange	RecA	hRad51	Forms nucleoprotein filaments; forms complexes with Rad52 and Rad54
Rad52	56,064	ssDNA binding and annealing	none	hRad52	Mediator of strand exchange; required for single strand annealing and BIR
Rad54	101,776	DNA-dependent ATPase	none	hRad54	Member of Snf2 family; promotes homologous DNA pairing by Rad51
Rad55	46,347	ssDNA binding	none	XRCC2, XRCC3, Rad51B, Rad51C, Rad51D	Forms heterodimer with Rad57; Rad55–Rad57 heterodimer functions as mediator in strand exchange
Rad57	52,242	ssDNA binding	none	XRCC2, XRCC3, Rad51B, Rad51C, Rad51D	Forms heterodimer with Rad55; Rad55–Rad57 heterodimer functions as mediator in strand exchange
Rad59	26,632	ssDNA binding and annealing	none	not known	Homology to Rad52; required for single strand annealing
Dmc1	36,606	ATP-dependent homologous DNA pairing[a]	RecA	hDmc1	Interacts with Rdh54/ Tid1 in two-hybrid system
Rdh54/Tid1	108,058	not known	none	hRad54	Member of Snf2 family; interacts with Dmc1 and Rad51 in two-hybrid system
RPA	70,339	ssDNA binding	none	hRPA	Removes secondary structure in ssDNA during the presynaptic phase of strand exchange
	29,921				
	13,810				

[a]Based on results with hDmc1 [52].

bles into a nucleoprotein filament on both ssDNA and dsDNA that is almost identical to the equivalent RecA-DNA nucleoprotein filament in overall dimensions and structure [65,98] and (ii) like RecA, Rad51 exhibits a homologous DNA pairing and strand exchange activity that yields joints between two DNA molecules [97].

Outside of the central homologous core of about 220 amino acids, RecA and Rad51 actually differ significantly, with Rad51 bearing an amino-terminal extension of about 120 amino acids, but is shorter than RecA by about 90 amino acids at the carboxyl-terminus. In addition, yeast Rad51 has a putative leucine zipper motif ($L–X_6–L–X_6–L–X_6–F$) from residues 296–317. These structural distinctions between RecA and Rad51 could account for the functional differences between the two proteins and could perhaps also reflect the unique sets of evolutionarily divergent recombination factors with which the two recombinases have to interact to accomplish their biological roles. Like RecA, Rad51 has a DNA-dependent ATPase activity. Maximal ATP hydrolysis

by Rad51 is seen with ssDNA, with duplex DNA being 5–10-fold less effective in activating ATP hydrolysis [97]. The kcat for ATP hydrolysis with ssDNA as cofactor is less than 1/min, which is about 40-fold lower than what has been observed for RecA [95,97].

Homologous DNA pairing and strand exchange mediated by Rad51. The homologous DNA pairing and strand exchange reaction has been studied in vitro using a variety of available systems, some of which are described in Fig. 3. The common feature of all of these in vitro systems is that there is a single DNA strand, which is the equivalent of the 3′ ssDNA tail generated via DNA end-processing in vivo, and a homologous duplex DNA molecule that is either linear or covalently closed, which can be considered the prospective DNA homolog, i.e. the homologous chromosome or sister chromatid. The reaction is initiated via the assembly a Rad51-ssDNA nucleoprotein complex, into which the homologous duplex DNA molecule is incorporated for DNA homology search and DNA joint formation with the single strand.

The reaction phase in which assembly of the Rad51-ssDNA nucleoprotein complex occurs is called the ''presynaptic'' phase, a process that is simple conceptually but in reality surprisingly complex, being dependent on the single-strand DNA binding factor RPA and molecular ''mediators'', as described in more detail below. Later, reaction steps which collectively lead to stable pairing between the recombining DNA molecules occur in the ''synaptic'' phase, which is also described in some detail below. Once a stable joint is formed between the two recombining DNA molecules, branch migration extends the length of the joint, resulting in formation of a substantial amount of heteroduplex DNA (see below).

Distinct phases of the homologous DNA pairing and strand exchange reaction

The presynaptic phase. In the presence of ATP, Rad51 polymerizes onto ssDNA [98] and dsDNA [65] to form helical nucleoprotein filaments that are right-handed [65]. Formation of the Rad51 filament on ssDNA is stimulated by the heterotrimeric ssDNA binding factor RPA [98], whereas Rad51 filament

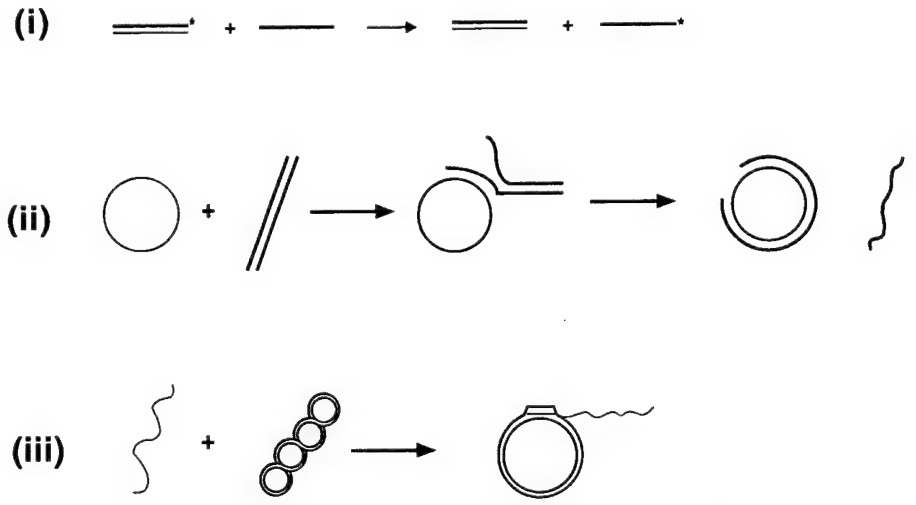

Fig. 3. Systems for studying the homologous DNA pairing and strand exchange reaction. (i) This prototypical system that uses oligonucleotides is quite sensitive, and it allows one the option of examining the influence of DNA sequence context on homologous DNA pairing. Because of the short length of the substrates, this system is not very useful for studying the strand exchange or branch migration reaction. (ii) This system employs the viral (+) strand and linear duplex form of DNA from a bacteriophage, and is used frequently for examining homologous DNA pairing and strand exchange by RecA and Rad51. (iii) To measure D-loop formation, one employs a linear single strand and a covalently closed duplex, normally a supercoiled molecule. The D-loop is the first DNA intermediate formed in vivo, and studies with yeast proteins have indicated that formation of D-loop requires Rad54, in addition to Rad51 and RPA (Petukhova et al., 1998). The readers can find more information on in vitro homologous DNA pairing and strand exchange systems in other review articles [50,81]. Portions of this figure were adapted from Camerini-Otero and Hsieh [15].

assembly on dsDNA shows no dependence on RPA [65,98]. The formation of Rad51 filament on either ssDNA or dsDNA requires ATP [65,98], although ATP binding alone appears to be sufficient for assembly [99]. The Rad51 nucleoprotein filament on circular dsDNA has been analyzed by three-dimensional reconstruction [65], and the results indicate that the Rad51 filament is almost identical in overall dimensions and appearance to the equivalent filament of RecA, with a pitch of 99 Å and 18.6 base pairs of DNA per helical turn. The most notable feature about the Rad51-dsDNA filament is that the DNA is held in a highly extended conformation, as reflected in the axial rise of 5.1 Å per base pair as compared to 3.4 Å per base pair encountered in normal B-form DNA [65]. The Rad51 filament formed on ssDNA has not been analyzed in as much detail, but it also shows the same extended conformation and shares similar overall dimensions as the Rad51-dsDNA filament [98].

Biochemical analyses have indicated that the homologous DNA pairing and strand exchange reaction occurs within the confines of the Rad51-ssDNA nucleoprotein filament, whereas the Rad51 filament on dsDNA is not capable of mediating this reaction [98]. The extent of Rad51 filament assembly on ssDNA can be followed by electron microscopy, but much more conveniently by simply measuring the level of ssDNA dependent ATPase activity [95]. Although RPA is important for the assembly of a contiguous filament on ssDNA and is therefore an important cofactor in the strand exchange reaction [95,97], an excess of RPA can in fact suppress this reaction [100]. This inhibitory effect of RPA has been attributed to competition with Rad51 for binding sites on the ssDNA. Two factors, Rad52 and Rad55–Rad57, help Rad51 overcome the competition posed by RPA, as described below.

The synaptic phase. Once the Rad51-ssDNA nucleoprotein filament is assembled, it is capable of taking up another DNA molecule, which could be a single-strand or a duplex. In this regard, the ssDNA molecule onto which the Rad51 filament has assembled may be viewed as being bound within a primary site within the filament, and the incoming homologous duplex molecule bound within a secondary site in the filament [50,81]. For stable pairing between the ssDNA and the duplex to occur, homologous contacts need to be established between the two recombining DNA molecules within the nucleoprotein filament. By reason of probability, the initial contact points between the ssDNA and the duplex are not at homologous locales. Exactly how the Rad51 nucleoprotein filament samples the incoming duplex to locate DNA homology is not known at this juncture. In the case of the RecA filament, it is believed that DNA homology search is relatively rapid and involves random collisions of the two DNA molecules [50,81]. In theory, binding of the duplex to the secondary site in the RecA filament has to be transient for a random collision mechanism to work efficiently. In support of this deduction, Mazin and Kowalczykowski [58] have provided evidence that the secondary binding site in the RecA filament indeed has only modest affinity for a duplex molecule. Experimental evidence does not support extensive sliding between the two DNA molecules within the RecA filament as a major means for locating DNA homology [3]. Given the precedent with RecA, it seems reasonable to propose that DNA homology search conducted by the Rad51-ssDNA nucleoprotein filament may primarily go through the random collision mode as well.

Once DNA homology is found, alignment of the two recombining molecules is established through a series of transient joints called paranemic joints (Fig. 4A). In RecA studies, the exact nature of the paranemic joint is still a subject of debate. Some have argued that the paranemic linkage involves a DNA triplex structure (i.e. all three strands are held together via novel non-Watson–Crick bonding), whereas other investigators believe that DNA strand switch occurs in the paranemic joint, with the extent of the strand switch being limited by topological constraints and dependent on the local nucleotide sequence context. Regardless of the true nature of the paranemic joint, it has been demonstrated in a number of studies, and although the paranemic joints dissociate readily when the RecA filament is disrupted (by deproteinization treatment, for instance), they are believed to be an important DNA intermediate that serves to capture the duplex and bring the two recombining DNA molecules into homologous registry [50,78,81].

When the two recombining DNA molecules are aligned, then there is the potential for the formation

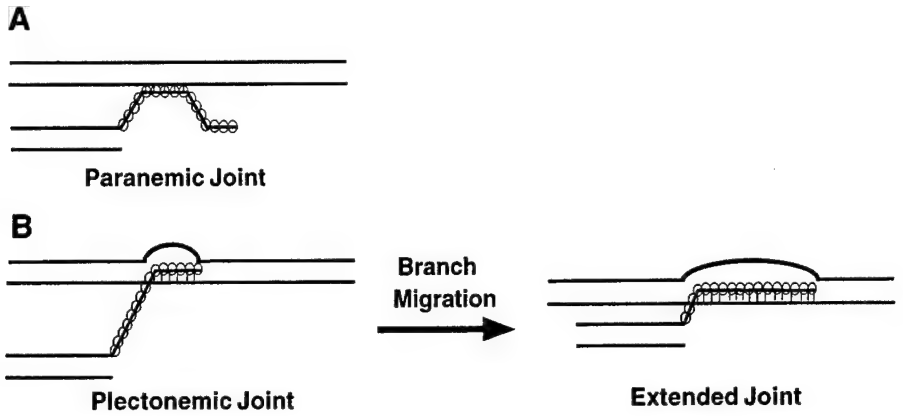

Fig. 4. DNA joints in the formation of heteroduplex DNA. (A) The first homologous joints formed between the recombining DNA molecules are paranemic. The paranemic joints are unstable but are believed to be important intermediates which lead to the formation of a stable joint molecule. The exact nature of the paranemic joint remains a subject of debate (see text and Roca and Cox [81]). (B) Once a free DNA end is located, the formation of a stable plectonemic linkage ensues. The reaction in which the plectonemic joint is extended is called branch migration or strand exchange. The circles in (A) and (B) denote Rad51 molecules. It should be noted that the nucleoprotein complex that conducts the reaction steps very likely contains other factors of the *RAD52* group, including Rad52, Rad54, and Rad55–Rad57 (see text).

of a stable joint molecule. This occurs when a free DNA end is present either in the ssDNA or the dsDNA molecule to allow for intertwining of the ssDNA strand with the complementary strand in the duplex partner. The plectonemic joint that results is much more stable than the paranemic linkage, because the two DNA molecules in the plectonemic joint are not only base-paired but are also topologically linked. In the branch migration reaction, the length of the plectonemic joint is extended (Fig. 4B). In the case of the Rad51 filament, branch migration proceeds 3′ to 5′ with respect to the initiating single strand, a reaction polarity opposite to that of RecA [98]. However, other results have suggested that branch migration within the Rad51 filament can also proceed in the 5′ to 3′ direction [63]. It is clear that branch migration mediated by the Rad51 filament does not need ATP hydrolysis [99], although based on the RecA model, one suspects that ATP hydrolysis could increase the bias for branch migration in a certain direction [81].

Recombination factors that function in the presynaptic phase

RPA. As alluded to above, the heterotrimeric ssDNA binding factor RPA is required for the efficient assembly of the Rad51 presynaptic filament, as verified by electron microscopy [98] and by examining the level of Rad51 ssDNA-dependent ATPase activity [95]. *E. coli* single-strand DNA binding protein (SSB) is as effective as yeast RPA in stimulating the ssDNA dependent ATPase and the DNA strand exchange activities of Rad51. Since Rad51 and SSB are not expected to interact physically, the results suggest that the major functional role of RPA in presynapsis is to remove the secondary structure in the ssDNA substrate [95]. This conclusion is directly supported by the observation that homopolymeric DNA species which are devoid of secondary structure are significantly more effective than natural ssDNA species in activating ATP hydrolysis by Rad51 [95].

In Rad51-mediated strand exchange, the stimulatory effect of RPA is seen most clearly when it is incorporated into the reaction after Rad51 has already nucleated onto the ssDNA. If an excess of RPA is added before or with Rad51 to the ssDNA substrate, suppression of strand exchange ensues [100]. RPA inhibits the ssDNA-dependent ATPase activity of Rad51 under conditions where it suppresses strand exchange [91,95]. Taken together, the results indicate that RPA can interfere with the assembly process by competing for binding sites on the

ssDNA substrate. Since RPA is at least as abundant as Rad51 in yeast cells, it seems likely that specific ancillary factors function with Rad51 in vivo to overcome the competition posed by RPA. Indeed, Rad52 and the Rad55–Rad57 complex have been shown to facilitate the assembly of the Rad51-ssDNA nucleoprotein filament when RPA is competing for DNA binding sites, as described in greater detail below.

Rad52, a mediator in strand exchange. RAD52 encoded product does not show obvious homology to any of the known recombination proteins in bacteria, and therefore appears to be unique to eukaryotes. Rad52 from yeast and humans binds to DNA as a ring-shaped multimer [92,109], showing higher affinity for ssDNA than dsDNA [61,92]. Recently, Van Dyck et al. [109] reported that human Rad52 binds specifically to DNA-ends.

Rad52 is of lower cellular abundance than Rad51, and forms a stable, stoichiometric, and co-immunoprecipitable complex with Rad51 [101]. Rad51–Rad52 complex has a mean size of greater than 1 MDa in gel filtration and its formation is not influenced by ATP (B.W. Song and P. Sung, unpublished results). Although Rad52 has been reported to interact with RPA in solution [92] and when RPA is bound to DNA [96], a stable complex between RPA and Rad52 cannot be demonstrated in cell extract or when purified Rad52 and RPA are mixed (B.W. Song and P. Sung, unpublished results). As described earlier, an excess of RPA added with Rad51 to the ssDNA substrate suppresses nucleoprotein filament assembly and compromises strand exchange efficiency. Under this circumstance, addition of Rad52 effectively overcomes the inhibition posed by RPA [64,92,100], and an amount of Rad52 approximately one tenth of Rad51 is already optimal for restoring strand exchange [101]. Although Rad52 is a ssDNA binding protein, it does not replace RPA in the strand exchange reaction [64,91,101]. Thus, Rad52 plays a highly specific role in the homologous pairing and strand exchange reaction, mediating a productive interaction between the recombinase Rad51 and the ssDNA binding factor RPA [101]. Because of this function of Rad52, it has been coined a "mediator" of DNA strand exchange [45,100]. How Rad52 exerts its mediator function is not known at present, but it seems reasonable to propose that Rad52 targets Rad51 to ssDNA, which can then act as the nucleation sites for filament growth.

Genetic studies have indicated that Rad52 also functions in at least two other pathways of recombination, namely, single-strand annealing (SSA) and DNA break induced replication (BIR), which are discussed in more detail below. The involvement of *RAD52* in multiple recombination pathways could be the reason why it appears to be the most important recombination factor in mitotic cells. Thus, although null mutations in *RAD52* essentially eliminate all the cellular ability to carry out recombination, it could be due to the cumulated effects of defects in different recombination pathways and does not necessarily mean that a certain recombination mechanism is more dependent on Rad52 than on other members of the *RAD52* group. Interestingly, the *RAD52* homolog in vertebrates does not appear to be nearly as indispensable in recombination and repair [80,112], suggesting that either Rad52 is not involved in as many different recombination pathways in higher organisms, or another recombination factor, a Rad52-homologous protein perhaps, provides parallel functions in other eukaryotes.

Rad55–Rad57 complex, and its mediator function. RAD55 and *RAD57* genes are unique among the *RAD52* group members in that their mutants, including null mutants, are cold sensitive for recombination and for sensitivity to ionizing radiation [55]. The recombination defects in the *rad55 rad57* double mutant are no more severe than those in single mutants, indicating a tight epistatic relationship between the two genes. Interestingly, Rad55 and Rad57 share some limited homology to RecA and Rad51, particularly in the sequence motifs involved in the binding of nucleoside triphosphates [54], and have in fact been referred to as RecA homologs. Rad55 and Rad57 interact in the yeast two-hybrid system [34,41]. In agreement with the two-hybrid results, Rad55 and Rad57 coimmunoprecipitate from cell extract and co-purify chromatographically. Sizing experiments revealed that the majority of Rad55–Rad57 is heterodimeric [101]. Interestingly, although Rad51 was shown to interact strongly with Rad55 in the yeast two-hybrid assay [34,41], Rad55–Rad57 complex does not co-immunoprecipitate with Rad51 from cell extract and purified Rad55–Rad57 heterodimer binds only weakly to Rad51 immobilized on Affi-gel beads

[100]. Mutations in the Walker type A nucleotide binding motif of Rad55 interfere with its recombination functions, whereas the equivalent mutations in Rad57 have little or no effect [41].

Addition of Rad55–Rad57 complex to the strand exchange reaction also effectively overcomes the competition posed by RPA, indicative of a mediator function of the heterodimer [101]. Like Rad52, the Rad55–Rad57 heterodimer is of lower cellular abundance than Rad51, and amounts of Rad55–Rad57 substoichiometric to that of Rad51 are already sufficient to effect the optimal level of mediator function. Although Rad55–Rad57 heterodimer has a ssDNA binding activity, it does not replace RPA in strand exchange and does not appear to possess strand exchange activity [101].

Since a mediator function has been identified in Rad52 as well [64,91,100], it is possible that Rad55–Rad57 heterodimer acts via a different mechanism or assists Rad51 at a stage in the assembly of the presynaptic filament temporally distinct from the reaction step that is dependent on Rad52. Alternatively, or in addition, Rad52 and Rad55–Rad57 complex may provide parallel, overlapping functions to ensure that the assembly of the Rad51 nucleoprotein filament occurs efficiently in vivo.

Recombination factors that function in the synaptic phase

Rad54. The *RAD54* encoded product belongs to the Swi2/Snf2 protein family, members of which are involved in diverse chromosomal processes including transcription, nucleotide excision repair, and post-replicative repair (reviewed in Ref. [25]). Consistent with the presence of Walker type nucleotide binding motifs in Rad54 [26], purified Rad54 has a robust ATPase activity (kcat $\sim 1000\ min^{-1}$) that is completely dependent on DNA, dsDNA in particular, for its activation. However, Rad54 does not possess a DNA helicase activity [72a]. Rad54 is a monomer in solution, but in the presence of DNA assembles into higher order species, as revealed by protein cross-linking [72]. Rad54 is of lower cellular abundance than Rad51 [40] and physically interacts with Rad51 [19,40,72a]. The addition of Rad54 to a homologous DNA pairing reaction consisting of circular ssDNA and linear duplex (systems (i) and (ii) in Fig. 3) results in strong stimulation of the homologous pairing rate [72a]. The first DNA intermediate predicted in the DNA DSB repair model for recombination is a D-loop structure formed between the initiating ssDNA tail and the DNA homolog (see Fig. 1). While Rad51 is incapable of mediating D-loop formation in vitro (system (iii) in Fig. 3), the inclusion of Rad54 renders D-loop formation possible [72a]. Rad54 by itself does not have homologous DNA pairing activity, nor does it replace RPA in this reaction [72a].

Rad54 from both yeast and humans mediates an alteration in duplex DNA conformation that results in a DNA linking number change [72,102]. This reaction has a strict dependence on ATP hydrolysis, as indicated from biochemical studies using the non-hydrolyzable ATP analog ATP-γ-S and substitution of Rad54 with mutant variants (hrad54 K189R, yrad54 K341A, and yrad54 K341R) that do not hydrolyze ATP [72,102]. Whether the DNA conformational change entails DNA strand separation is not known at this point. It seems reasonable to propose that the ability to alter DNA conformation in a manner that is dependent on ATP hydrolysis is a conserved property of Rad54, and that this property is germane for the recombination function of Rad54.

The *rad54 K341A* and *rad54 K341R* alleles, which habor mutations in the highly conserved lysine residue in the Walker type A sequence, have been shown to encode mutant rad54 proteins defective in ATP hydrolysis [72]. Clever et al. [19] found that whereas overexpression of wild type *RAD54* gene suppresses the ultraviolet and MMS sensitivities of a *rad51Δ* mutant, overexpression of the *rad54 K341R* allele has no such effect. The *rad54 K341R* allele in a *rad54Δ* background is also defective in the repair of DNA lesions induced by MMS, intrachromosomal gene conversion in haploid *rad54Δ* cells, and in meiosis. The *rad54 K341A* mutant gene behaves like the *rad54 K341R* allele phenotypically [72]. Taken together, it seems clear that the Rad54 ATPase function is indispensable for *RAD54* dosage-dependent suppression of the DNA repair defects of the *rad51Δ* mutation [19] and also for different types of mitotic and meiotic recombination [72].

The *rad54* null mutant is not as affected in diploid interchromosomal gene conversion as in haploid gene conversion [48]. One possible explanation is that the *RAD54* related gene *RDH54/TID1* (see

below) provides the ability to carry out most of the diploid interchromosomal gene conversions, with only a small fraction being effected through *RAD54* ([48]; see below). Interestingly, diploid mitotic gene conversion was not significantly decreased in the *rad54K341A* and *rad54K341R* mutants [72]. This result suggests that either *rad54K341A* and *rad54K341R* mutants could promote diploid mitotic gene conversion on their own, or Rad54, but not its ATPase activity, is required for the integrity of a higher order complex important for gene conversion in diploid cells [72]. Since many members of the Swi2/Snf2 family of proteins function to remodel chromatin, it is an open possibility that Rad54 also has a chromatin remodeling function.

Rdh54/Tid1. Based on computer search, a homolog of *S. cerevisiae RAD54*, *RDH54*, has been identified [48,90]. *RDH54* was independently isolated as a gene whose product interacts with the meiosis-specific Rad51 homolog Dmc1 in a yeast two-hybrid screen, and was named *TID1* [24]. Rdh54/Tid1 also interacts with Rad51 in the two-hybrid assay, albeit with a lower apparent affinity than with Dmc1 [24]. Rdh54 shows about 35% sequence identity to Rad54. Although the *rdh54Δ* mutation confers only slightly sensitivity to MMS in haploid cells, it greatly sensitizes the MMS sensitivity of a *rad54Δ* haploid strain. Likewise, the *rad54Δ rdh54Δ* double mutant is more impaired in meiosis than either single mutant alone [48,90]. Interestingly, diploid yeast strains harboring homozygous deletions of *RAD54* and *RDH54* are severely growth retarded, and this impairment can be overcome by simultaneously deleting *RAD51*, strongly suggesting that the growth deficiency stems from attempted, but incomplete recombination. A diploid strain harboring homozygous deletions of *RDH54* and *SRS2* is inviable, and the lethality is also overcome by simultaneously deleting *RAD51* [48].

Superficially, it might appear that Rdh54 is simply providing a recombination function redundant to that of Rad54 during mitotic growth. However, Klein [48] discovered that diploid *rdh54Δ* cells are much more defective in interchromosomal gene conversions than diploid *rad54Δ* cells, indicating a specialized function of Rdh54 in interchromosomal recombination. Whether this specificity stems from a unique ability of Rdh54 to interact with proteins required for interchromosomal recombination, or because Rdh54 provides a specific enzymatic function during interchromosomal recombination, remains to be determined.

Given the structural similarity of Rdh54 to Rad54 and its involvement in recombination processes, it is a distinct possibility that Rdh54 also possesses biochemical functions similar to what have been reported for Rad54 [72,72a]) and affects heteroduplex DNA formation by a similar mechanism. The interaction of Rdh54 with Dmc1 and Rad51 could mean that Rdh54 functions with both recombinases to promote heteroduplex DNA formation. Whether Rdh54 has a chromatin remodeling function is an interesting possibility that needs to be tested.

Other recombination factors

Rad59. Bai and Symington [6] identified a mutant, called *rad59*, which lowers the level of intrachromosomal recombination in a *rad51* mutant background. The *rad59* mutant exhibits sensitivity to γ-ray, which was used as the basis for cloning the *RAD59* gene. *RAD59* has a meiotic function, as indicated by a synergistic decline in sporulation efficiency when combining a leaky mutation in *RAD52*, *rad52 R70K*, with the *rad59Δ* mutation [7]. Interestingly, Rad59 shows homology to the amino-terminal region of Rad52, and overexpression of Rad52 suppresses the γ-sensitivity of the *rad59* mutant [6]. More recently, both the Symington group [7] and the Haber group (personal communication) have found that Rad59 is required for recombination by SSA. Rad59 has a ssDNA binding activity and, consistent with its involvement in SSA, mediates the annealing of complementary DNA strands in vitro [73]. Bai and Symington [6] have envisioned that Rad59 functions in the context of a complex with Rad51, Rad52, and other recombination factors to ensure that ssDNA substrates are channeled efficiently into recombination pathways, and that Rad52 together with Rad59 may have the ability to promote strand invasion [7]. These ideas need to be tested with purified proteins.

Dmc1. The yeast *DMC1* encoded product is homologous to RecA [13,94] and much more so to Rad51 [13]. *DMC1* gene is required for normal levels of meiotic recombination and is therefore important for chromosomal disjunction during meiosis I. The expression of *DMC1* is restricted to meiosis, and consistent with this expression pattern, a *dmc1Δ*

mutation produces no discernible mitotic phenotype [13]. From gene knockout experiments in mice, it is clear that the Dmc1 homolog in mammals also has important meiotic functions [75,113]. Human Dmc1 has been purified and shown by Li et al. [52] to possess homologous DNA pairing activity. Dmc1 forms octameric rings, which stack on DNA, but does not apparently form a helical nucleoprotein filament [57,67].

Cell biological tool for studying recombination. The use of cell biological techniques to study recombination in yeast is a relatively new and exciting development. Because of the availability of yeast mutants defective in various stages of recombination processes, their analyses are often revealing as to their effect on the assembly of recombination protein complexes. The patterns of nuclear redistribution of various recombination factors during meiosis and following DNA damaging treatment have been examined [12,30,56,108]. For instance, the assembly of meiotic nuclear foci of Rad51 has been shown to be dependent on *RAD52*, *RAD55*, *RAD57*, and on genes that control the formation of meiotic DSBs [30], results which very nicely corroborate mechanistic predictions based on genetic and biochemical analyses. Currently, the cell biological approach is as close as one can get to visualizing recombination processes in situ. This approach will continue to provide valuable information concerning the temporal sequence and genetic requirements for the assembly of higher order recombination protein complexes in vivo.

Repair DNA synthesis and resolution of recombination intermediates. Based on the observation that during conversion of the mating type information at *MAT*, the repair DNA synthesis step requires the concerted action of DNA polymerases α, δ, and ε, Holmes and Haber [37] have suggested that the repair synthesis reaction entails the establishment of both leading and lagging DNA strands. Whether the results of Holmes and Haber on mating type switching apply to other recombination processes and how the various DNA synthesis factors are recruited to sites of recombination are interesting subjects that need to be addressed.

Two genes, *MSH4* and *MSH5*, which encode proteins with considerable homology to the mismatch repair factors Msh2 and Msh3, are required for wild type levels of crossover recombination during meiosis [36,83]. In the *msh4* and *msh5* mutants, the levels of meiotic gene conversion and post meiotic segregation appear to be normal at the majority of the loci examined, indicating no overt defect in mismatch repair in these mutants. Because of the deficiency in crossover recombination, *msh4* and *msh5* mutants exhibit a defect in chromosome disjunction during meiosis I, resulting in a sporulation deficit and low spore viability. The *MSH4* and *MSH5* genes are epistatic to each other in meiotic crossover recombination, consistent with the suggestion that their encoded products function in the same biological pathway or reaction [36], and also with the observation that the two proteins are associated as a stable complex [77]. Other results have indicated a role for the mismatch repair protein Mlh1 in the Msh4–Msh5 dependent pathway of meiotic crossover recombination [38]. Since Msh proteins have the ability to bind DNA mismatches and specific DNA structures [4], it is possible that Msh4–Msh5 complex in conjunction with Mlh1 may specifically recognize and stabilize a DNA intermediate, such as the Holliday junction, critical for the formation of crossover recombinants. Expression of *MSH4* and *MSH5* is restricted to meiosis, and in yeast strains mutated for these two genes, no mitotic phenotypes can be discerned [36,83].

Aside from a possible function of the Msh4–Msh5 complex in Holliday junction recognition and perhaps its stabilization, little is known about other recombination factors that promote branch migration of Holliday junctions. Although a mitochondrial Cruciform Cutting Endonuclease, CCE1, has been described [86], the resolvases that process Holliday junctions and other DNA intermediates in nuclear chromosomal recombination have not been identified.

2.2. Recombination by SSA

As the name implies, this mechanism involves the annealing or hybridization of two complementary DNA single strands to yield a recombinant. SSA has mostly been studied using plasmid or chromosomal constructs that carry direct repeats of a genetic element, but the work of Haber and Leung [33] has indicated that SSA can in fact occur across chromo-

somes. Genetically, SSA is less complex than classical recombination, being dependent on *RAD52* [66] and *RAD59* ([7]; Jim Haber, personal communication), but the other *RAD52* group members are apparently dispensable.

Consistent with the genetic data implicating *RAD52* in SSA, Rad52 anneals complementary DNA strands in vitro [61]. Interestingly, single strand annealing by Rad52 is stimulated by RPA [92,96], which is thought to remove secondary structure in the single strands and directs Rad52 to the bound single strands via specific protein–protein interactions [96]. As alluded to earlier, Rad59 possesses an ability to bind ssDNA and anneals complementary DNA strands. However, DNA annealing by Rad59 does not appear to depend on RPA [73]. In fact, relatively high concentrations of RPA inhibit the Rad59-mediated DNA strand annealing reaction. Rothstein et al. previously isolated a mutant of the *RFA1* gene which encodes the largest subunit of RPA and found that this mutation, *rfa1-D228Y*, allows SSA in a *rad52* mutant background. Interestingly, SSA in the *rfa-D228Y* mutant occurs much more frequently than in the isogenic wild type strain, which suggests that a normal level of RPA in fact suppresses SSA in vivo [93].

In addition to Rad52 and Rad59, genetic studies have implicated the DNA structure-specific endonuclease Rad1–Rad10 in trimming the non-homologous, unhybridized ssDNA overhangs during SSA (see Fig. 5). Interestingly, the mismatch repair factors Msh2 and Msh3 also appear to play a role in some SSA events, and it is possible that these factors serve to stabilize the annealed DNA structure and to target the Rad1–Rad10 endonucleolytic activity to the ssDNA overhangs [66]. It is not known whether the single-strand gaps after trimming of ssDNA overhangs are filled by a particular DNA polymerase. In addition to an involvement in SSA, Rad1 and Rad10 also seem to play a role in other mitotic recombination events [39,85]. Interestingly, the Rad1 homolog in Drosophila, Mei-9, is important for meiotic recombination as well [87].

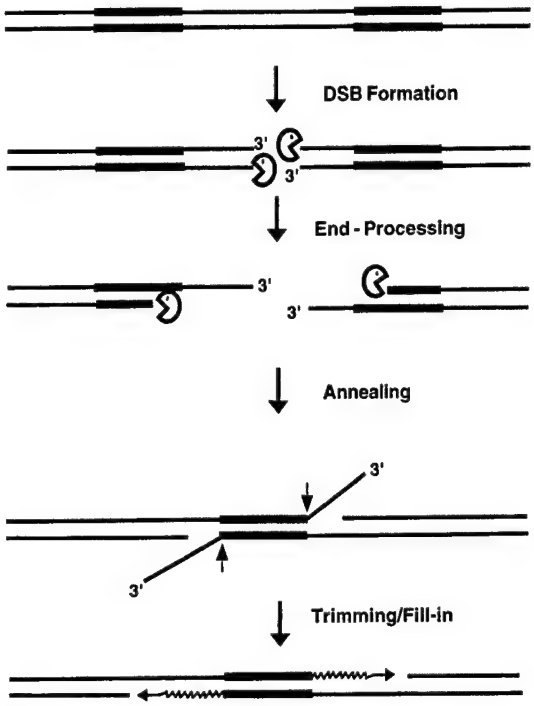

Fig. 5. Conceptual model for recombination by SSA. A DSB formed between direct repeats (represented by the dark lines) of a genetic element is processed exonucleolytically to yield 3′ ssDNA tails. Because of the presence of DNA homology, the DNA tails can anneal to each other to form a joint. After strand annealing, nucleolytic trimming of the overhanging ssDNA tails and fill-in by a DNA polymerase yield a recombinant that has some of the original DNA sequence deleted.

2.3. Recombination by BIR

A pathway of very long tract gene conversion has been described, which entails the formation of a short DNA joint between an initiating ssDNA substrate and a DNA molecule at a site where there is localized homology between the DNA molecules, followed by DNA synthesis to copy information contained within the DNA homolog. This pathway of recombination is dependent on *RAD52*, but not on *RAD51* [66]. It is possible that the DNA strand annealing activity of Rad52 is germane for establishing the initial DNA joint to prime DNA synthesis during BIR. Given the similarities between Rad52 and Rad59, it is tempting to speculate that perhaps BIR is also dependent on *RAD59* gene. The manner in which DNA joint formation and DNA synthesis are coupled in BIR and the relative contributions of the various DNA polymerases in the DNA synthesis reaction during BIR remain to be determined.

2.3.1. Some major unresolved problems

2.3.1.1. Meiotic DNA DSB formation. It remains a real mystery how the meiotic cell decides when to order the introduction of DNA DSBs at various "hotspots". Is this process controlled by the synthesis of a critical protein factor at a certain stage of the meiotic program, or is it due to post-translational modifications of preexisting factors? Likewise, the mechanism by which Spo11, Rad50, Mre11, Xrs2, and other protein factors function to make the meiotic DNA DSBs is completely unknown at this point. Addressing these issues will require a combination of genetic, cell biological, and biochemical analyses.

2.3.2. Channeling of DSBs into recombination or non-recombination pathways

How is the decision made at the cellular level for channeling a DNA DSB into a certain recombination pathway or into non-homologous DNA end-joining? Is it possible that this decision is influenced by the cell cycle stage or is dependent on post-translational modifications of key protein components? Given that the trio of Rad50, Mre11, and Xrs2 are involved in both homologous recombination and non-homologous DNA end-joining, could they have a role in executing the cellular command to conduct either recombination or end-joining? These are interesting questions that again can be addressed genetically and biochemically.

2.3.3. Coupling of steps in recombination

It seems plausible that DNA end-processing, heteroduplex joint formation, and DNA synthesis are not distinct steps that occur independently, but rather that they are coupled to one another. This idea predicts a hierarchy of functional and physical interactions among factors traditionally thought of being required only in one or the other step of recombination. This idea can be tested, now that purified recombination factors are becoming available.

2.3.4. Chromatin structure

How does the recombination machinery deal with chromatin packing when conducting its business? The initiating ssDNA substrate that triggers recombination can be as long as 1 kb or more. Assuming that all of this ssDNA is utilized for heteroduplex DNA formation, then an extensive region of chromatin probably needs to be remodeled to allow strand invasion, branch migration, and subsequent reactions to occur. How is chromatin remodeling mediated during recombination and repair? Do Rad54 and Rdh54/Tid1 play a role in chromatin remodeling?

2.3.5. Recombination in higher organisms

From the phenotypes of other eukaryotic cells mutated for recombination genes, from animal models, and from biochemical analyses of human recombination factors, it is apparent that the functions of the *RAD52* group genes have been highly conserved. However, it is becoming clear that the genetic requirements for recombination are more complex and subject to additional layers of control in higher organisms. For instance, five Rad51-homologous proteins, Rad51B [5,79], Rad51C [23], Rad51D [76], XRCC2 [17,53], and XRCC3 [53] have already been identified in human cells. Evidence that at least some of these Rad51-homologous proteins have a role in recombination and repair has come from analysis of Chinese hamster ovary cell lines irs1 and irs1SF, which are, respectively, defective in *XRCC2* and *XRCC3* [44,105,104]. These cell lines show a marked deficiency in the repair of DNA DSBs through recombination. Transient transfection of XRCC2 and XRCC3 restores recombinational repair to near wild-type levels [42,74]. XRCC3 interacts with Rad51 in the yeast two-hybrid system and co-immunoprecipitates with Rad51 from cell extract [53], while Rad51C interacts with both XRCC3 and Rad51B in two-hybrid studies [23]. XRCC3 has also been shown in the Bishop laboratory to be required for the formation of DNA damage-induced Rad51 nuclear foci [14]. Given what we know about yeast Rad55 and Rad57, which are also homologous to Rad51, it seems possible that Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3 also form complexes with one another and function to enhance hRad51-ssDNA nucleoprotein filament assembly. It is not known whether these human Rad51-homologous proteins are all required simultaneously for efficient recombination or whether they play specific recombination roles in certain cell types or at certain cell cycle or developmental stages.

There is growing evidence that the recombination machinery is subjected to modulation by genes that have a tumor suppression function. In addition to *NBS1*, which encodes an integral component of the Mre11-associated nuclease complex as described at the beginning of this article, recent work has implicated the gene mutated in AT (*ATM*) and the breast tumor suppressor genes *BRCA1* [62] and *BRCA2* in modulating the efficiency of recombination. Specifically, BRCA1 has recently been shown to associate with the hRad50–hMre11–Nbs1 complex and colocalizes with hRad50 in discrete nuclear foci upon irradiation [114]. In addition to its checkpoint function, ATM may be directly involved in DNA repair processes, as AT cells remain hypersensitive to ionizing radiation under conditions where the checkpoint function is dispensable [103]. AT patients and all Atm-deficient mice are infertile due to the absence of mature gametes (reviewed by Ref. [51]). Detailed analysis of Atm-deficient mice indicates that ATM is required for an early stage of meiosis [8].

Several lines of recent evidence have implicated the breast tumor suppressor *BRCA2* in DNA repair by recombination. Cultured cells become sensitive to γ-irradiation upon down-regulation of BRCA2 [1], and the pancreatic adenocarcinoma Capan-1 cells, which lack one copy of the *BRCA2* gene and contain a truncating mutation (617delT) in the other *BRCA2* allele [31], are hypersensitive to various DNA damaging agents. In addition, fibroblasts derived from mutant mouse embryos deleted for *Brca2* are specifically sensitive to γ-irradiation [21,59,68]. Most importantly, several laboratories have reported the association of BRCA2 with Rad51, and the interaction domain has been mapped to the BRC repeat in BRCA2 [18,46,111]. These observations appear to point to a role for BRCA2 in recombinational repair by influencing the activities of Rad51.

3. Epilogue

In every measurable way, *S. cerevisiae* has served as an excellent model for learning about the genetics and biochemistry of recombination processes, and there are good reasons to believe that studies in this organism will continue to yield answers to fundamental questions concerning recombination. The emergence of various tumor suppressors as potential modulators and regulators of recombination processes in mammals is an exciting recent development that has captivated investigators who otherwise work outside of the recombination field. Aficionados of recombination mechanisms can now legitimately declare that what they have loved to study is not only fascinating, but is also germane for human health.

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Basis for Avid Homologous DNA Strand Exchange by Human Rad51 and RPA*

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Human Rad51 (hRad51), a member of a conserved family of general recombinases, is shown here to have an avid capability to make DNA joints between homologous DNA molecules and promote highly efficient DNA strand exchange of the paired molecules over at least 5.4 kilobase pairs. Furthermore, maximal efficiency of homologous DNA pairing and strand exchange is strongly dependent on the heterotrimeric single-stranded DNA binding factor hRPA and requires conditions that lessen interactions of the homologous duplex with the hRad51-single-stranded DNA nucleoprotein filament. The homologous DNA pairing and strand exchange system described should be valuable for dissecting the action mechanism of hRad51 and for deciphering its functional interactions with other recombination factors.

Genetic studies in various eukaryotic organisms have indicated that homologous recombination processes are mediated by a group of evolutionarily conserved genes known as the *RAD52* epistasis group. As revealed in studies on meiotic recombination and mating type switching in *Saccharomyces cerevisiae*, DNA double-strand breaks are formed and then processed exonucleolytically to yield long single-stranded tails with a 3′ extremity. Nucleation of various *RAD52* group proteins onto these ssDNA[1] tails renders them recombinogenic, leading to the search for a homologous DNA target (sister chromatid or homologous chromosome), formation of DNA joints with the target, and an exchange of genetic information with it. The repair by recombination of DNA double-strand breaks induced by ionizing radiation and other DNA damaging agents very likely follows the same mechanistic route, as it too is dependent on genes of the *RAD52* epistasis group (reviewed in Refs. 1 and 2).

Among members of the *RAD52* group, the *RAD51*-encoded product is of particular interest because of its structural and functional similarities to the *Escherichia coli* recombination protein RecA (2–5). RecA promotes the pairing and strand exchange between homologous DNA molecules to form heteroduplex DNA (4, 5), an enzymatic activity believed to be germane for the central role of RecA in recombination and DNA repair processes. Likewise, homologous DNA pairing and strand exchange activities have been shown for *S. cerevisiae* Rad51 (yRad51) (6). Under optimized conditions, the length of heteroduplex DNA joints formed by yRad51 and RecA can extend over quite a few kilobase pairs (4, 5, 7).

In published studies, human Rad51 (hRad51) was found to have the ability to make DNA joints but the maximal potential for forming only about 1 kilobase pairs of heteroduplex DNA (8–11). Furthermore, while yRad51 and RecA require their cognate single-strand DNA binding factors, SSB and yRPA, for optimal recombinase activity, hRPA has been suggested to stimulate the hRad51-mediated homologous pairing and strand exchange reaction only when the hRad51 concentration is suboptimal (9, 10).

Given the central role of hRad51 in recombination processes and the fact that the activities of hRad51 are apparently subject to modulation by tumor suppressor proteins such as BRCA2 (reviewed in Ref. 12), establishing an efficient hRad51-mediated DNA strand exchange system will be important for dissecting the functional interactions among hRad51, other recombination factors, and tumor suppressors. In this work, a variety of reaction parameters that could influence the recombinase activity of hRad51 were explored. We demonstrate that under certain conditions, hRad51 makes DNA joints avidly and promotes highly efficient strand exchange over at least 5.4 kilobase pairs. Importantly, under the new reaction conditions, the efficiency of the hRad51-mediated DNA strand exchange reaction is strongly dependent on hRPA over a wide range of Rad51 concentrations tested.

EXPERIMENTAL PROCEDURES

DNA Substrates—φX174 viral (+)-strand was purchased from New England Biolabs and φX174 replicative form I DNA was from Life Technologies, Inc. The replicative form I DNA was linearized with *Apa*LI. The pBluescript DNA was prepared from *E. coli* XL-1 Blue (Stratagene), purified by banding in cesium chloride gradients, and linearized with *Bsa*I. The oligonucleotides (83-mer) used in strand exchange were: oligo 1, 5′-AAATGAACATAAGATAAATAAGTATAAGGATAATACAAAATAAGTAAATGAATAAACATAGAAAATAAAGTAAAGGATATAAA; oligo 2, the exact complement of oligo 1. Oligo 2 was labeled at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase and then annealed to oligo 1. The labeled duplex was purified from 10% polyacrylamide gels by overnight diffusion at 4 °C into TAE buffer (40 mM Tris acetate, pH 7.4, 0.5 mM EDTA). DNA substrates were stored in TE (10 mM Tris-HCl, pH 7.5, with 0.5 mM EDTA).

Plasmids—Plasmid pRh51.1 consists of human *RAD51 K313* cDNA under the control of the T7 promoter in vector pET11 (Novagen). Plasmid pRh51.1 was then subject to *in vitro* mutagenesis using the QuikChange kit (Stratagene), to change Lys^{313} (AAA codon) to a glutamine residue (CAA codon). The resulting plasmid, pRh51.2, was sequenced to ensure that no other undesired change has occurred in the *RAD51* sequence. Plasmid p11d-tRPA (13), which coexpresses all three subunits of hRPA, was used for purification of this factor.

Cell Growth—Plasmids pRh51.1 and pRh51.2 were introduced into the RecA-deficient *E. coli* strain BLR (DE3) with pLysS. Following transformation, single clones were picked and grown for 15 h in 30 ml of Luria broth. The starter culture was diluted 200 times with fresh

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[1] The abbreviations used are: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

Luria broth and incubated at 37 °C. When the A_{600} of the cultures reached 0.6 to 1, isopropyl-1-thio-β-D-galactopyranoside was added to 0.4 mM and the induction of hRad51 continued at 37 °C for 4 h. Cells were harvested by centrifugation and stored frozen at −70 °C. Plasmid p11d-tRPA was introduced into *E. coli* strain BL21 (DE3) and the induction of hRPA was carried out as described previously (13).

Protein Purification—All the following steps were carried out at 4 °C. For the purification of hRad51 Lys^{313} and hRad51 Gln^{313} proteins, *E. coli* cell paste, 30 g from 20 liters of culture, was suspended in 150 ml cell breakage buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 200 mM KCl, 2 mM dithiothreitol, 10% sucrose, and the following protease inhibitors: aprotinin, chymostatin, leupeptin, and pepstation A at 3 μg/ml each, and 1 mM phenylmethylsulfonyl fluoride) and then passed through a French press once at 20,000 p.s.i. The crude lysate was clarified by centrifugation (100,000 × *g*, 120 min), and the supernatant (Fraction I) was treated with ammonium sulfate at 0.23 g/ml to precipitate hRad51 and about 20% of the total extract protein. The ammonium sulfate pellet was dissolved in 300 ml of T buffer (30 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 mM EDTA, 0.5 mM dithiothreitol) with the set of protease inhibitors used in extract preparation, and then clarified by centrifugation (10,000 × *g* for 30 min). The cleared protein solution (Fraction II) was then applied onto a column of Q-Sepharose (2.6 × 6 cm; total 30-ml matrix) equilibrated in T buffer with 100 mM KCl and eluted with a 400 ml of gradient of 100 to 600 mM KCl in T buffer. The peak of hRad51 (Fraction III), eluting at about 330 mM KCl (60 ml), was dialyzed against T buffer with 100 mM KCl and then fractionated in a column of Affi-Gel Blue (Bio-Rad; 1.6 × 5 cm; total 10-ml matrix) with a 100-ml gradient of 100 to 2000 mM KCl in T buffer. The hRad51 protein eluted from Affi-Gel Blue at 800 to 1200 mM KCl, and the pool of which (Fraction IV; 20 ml) was dialyzed against T buffer with 100 mM KCl and fractionated in a column of Macro hydroxyapatite (Bio-Rad; 1 × 7.5 cm; total 6-ml matrix) with a 100-ml 30 to 320 mM KH_2PO_4 gradient in buffer T. hRad51 eluted from 150 to 220 mM KH_2PO_4, and the peak fractions were pooled (Fraction V; 15 ml containing 7.5 mg of hRad51), dialyzed against T buffer with 50 mM KCl, and applied onto a Mono S column (HR5/5), which was developed with a 30-ml 100 to 400 mM KCl gradient in buffer T. The Mono S fractions containing the peak of hRad51, eluting at about 250 mM KCl, were pooled (Fraction VI; 4 ml containing 6 mg of hRad51), diluted with an equal volume of 10% glycerol and then fractionated in Mono Q (HR 5/5) with a 30-ml 100 to 600 mM KCl gradient. The final pool of hRad51 (Fraction VII; 3 ml containing 5 mg of hRad51 in ~350 mM KCl) was concentrated in Centricon-30 microconcentrators and stored at −70 °C. The hRad51 concentration was determined using the calculated molar extinction coefficient of 12,800 M^{-1} cm^{-1} at 280 nM (10).

For the purification of hRPA, extract was made from *E. coli* BL21 (DE3) harboring the plasmid p11d-tRPA (13) and subjected to the purification procedure we have used for yRPA (14). The concentration of hRPA was determined by comparison of multiple loadings of hRPA against known amounts of bovine serum albumin and ovalbumin in a Coomassie Blue R-stained polyacrylamide gel.

DNA Strand Exchange System That Uses φX174 DNA—All the reaction steps were carried out at 37 °C. In Fig. 2, the reaction (50 μl final volume) was assembled by mixing hRad51 (7.5 μM) added in 2 μl of storage buffer and φX174 viral (+)-strand (30 μM nucleotides) added in 2 μl in 40 μl of buffer R (40 mM Tris-HCl, pH 7.8, 2 mM ATP, 1 mM $MgCl_2$, 1 mM dithiothreitol, and an ATP regenerating system consisting of 8 mM creatine phosphate and 28 μg/ml creatine kinase). After a 5-min incubation, hRPA (2 μM) in 2 μl of storage buffer was added, followed by a 5-min incubation, and then 5 μl of ammonium sulfate (1 M stock, final concentration of 100 mM), followed by another 1-min incubation. To complete the reaction, linear φX174 replicative form I DNA (30 μM nucleotides) in 3 μl of TE and 4 μl of 50 mM spermidine (4 mM) were incorporated. At the indicated times, 4.5-μl portions were withdrawn, mixed with 7 μl of 0.8% SDS and 800 μg/ml proteinase K, incubated for 15 min before electrophoresis in 0.9% agarose gels in TAE buffer. The gels were stained in ethidium bromide (2 μg/ml in H_2O) for 1 h, destained for 12 to 18 h in a large volume of water, and then subjected to image analysis in a NucleoTech gel documentation station equipped with a CCD camera using Gel Expert for quantification of the data. Unless stated otherwise, the reaction mixtures in other experiments were assembled in the same manner with the indicated amounts and order of addition of reaction components, except that they were scaled down two and one-half times.

DNA Strand Exchange System That Employs Oligonucleotides—The reaction mixture had a final volume of 12.5 μl and the steps were carried out at 37 °C. hRad51 (7.5 μM) was incubated with oligonucleotide 2 (30 μM nucleotides) in 10 μl of buffer R. The reaction mixture was completed by adding ammonium sulfate in 1 μl, 1 μl of 50 mM spermidine, and the radiolabeled duplex (30 μM nucleotides) in 0.5 μl. At all the times indicated, a 3-μl portion of the reaction mixture was deproteinized as described above and then subjected to electrophoresis in 10% polyacrylamide gels run in TAE buffer. The level of DNA strand exchange was determined by PhosphorImager analysis of the dried gels.

Examination of Interaction between hRad51-ssDNA Filament and Duplex DNA—Oligonucleotides F1 and F1b (Midland) both have the sequence 5′-TGGCTTGAACGCGTCATGGAAGCGATAAAACTCTGCA-GGTTGGATACGCCAATCATTTTTATCGAAGCGCGCCGCCC-3′, except that the latter also contains a biotin molecule positioned at the 5′ terminus. In these oligonucleotides, nucleotide residues 11 to 72 are complementary to positions 5348 to 23 of the φX (+)-strand DNA. These oligonucleotides were hybridized to φX (+)-strand by incubating a 3 M excess of the oligonucleotide with the latter in buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, and 1 mM dithiothreitol. The F1-φX (+)-strand and F1b-φX (+)-strand hybrids (30 μM nucleotides) were mixed with 10 μl of magnetic beads containing streptavidin (Roche Molecular Biochemicals) in binding buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM KCl, and 1 mM EDTA for 10 min at 37 °C. About 70% of the F1b-(+)-strand hybrid was immobilized on the beads, whereas, as expected, less than 5% of the F1-(+)-strand hybrid was retained. To assemble hRad51 filament on the immobilized φX (+)-strand, magnetic beads preloaded with the F1b-φX (+)-strand hybrid were incubated with 4 μM hRad51 in 20 μl of buffer R. Reproducibly, ~85% of the hRad51 was bound to the magnetic beads under the stated conditions, as determined by eluting the bound hRad51 with 2% SDS followed by SDS-polyacrylamide gel electrophoresis and staining with Coomassie Blue; this procedure gave an immobilized hRad51-ssDNA nucleoprotein complex of ~3 nucleotides/hRad51 monomer. The magnetic beads containing hRad51-ssDNA complex was then washed once each with 20 μl of buffer R with 0.01% Nonidet P-40 and 20 μl buffer R, before being incubated with linear φX duplex (8 μM nucleotides) for 3 min at 37 °C in 20 μl of buffer R containing 4 mM spermidine and the indicated concentrations of ammonium sulfate. The beads were treated with 20 μl of 2% SDS at 37 °C for 5 min to elute bound duplex and hRad51. The supernatants and SDS eluates were analyzed in agarose gels followed by staining with ethidium bromide to quantify DNA and in polyacrylamide gels with Coomassie Blue staining to determine the amount of hRad51. As controls, magnetic beads alone, magnetic beads preincubated with the F1-φX (+)-strand hybrid, and magnetic beads preincubated with F1b but without φX (+)-strand were similarly incubated with the linear φX duplex and then processed for analyses.

RESULTS

Recombination Factors—The cDNA for hRad51 was amplified from a human B-cell cDNA library. Sequencing of the *hRAD51* cDNA insert revealed that it was identical to one of the published *hRAD51* sequences (15) but differed from the other sequence (16) at amino acid residue 313; the former has a lysine (an AAA codon) while the latter has a glutamine (a CAA codon) at this position. The cloned cDNA was subjected to targeted mutagenesis to change lysine 313 to glutamine. Both hRad51 Lys^{313} and hRad51 Gln^{313} variants were expressed in *E. coli* and purified to near homogeneity (Fig. 1*A*). The two hRad51 isoforms behaved identically during purification and gave indistinguishable results in all the enzymatic assays described here. Only the results with hRad51 Lys^{313} are shown. We presume that the two hRad51 isoforms correspond to naturally occurring polymorphic variants. For DNA strand exchange experiments, the human ssDNA binding factor replication protein A (hRPA), a heterotrimer of 70-, 32-, and 14-kDa subunits, was also purified to near homogeneity (Fig. 1*B*) from *E. coli* cells harboring a plasmid which coexpresses all three subunits of this factor (13).

System for ATP-dependent Homologous DNA Pairing and Strand Exchange—For characterizing the homologous DNA pairing and strand exchange activity of hRad51, we used as substrates φX 174 viral (+)-strand and linear duplex that are 5.4 kilobase pairs in length (Ref. 6; schematic shown in Fig. 2*A*). In this system, hRad51 is preincubated with the (+)-strand, followed by the addition of hRPA, and the linear duplex

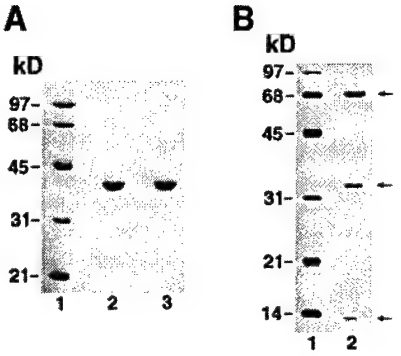

FIG. 1. **Recombination factors.** *A*, purified hRad51 Lys^{313} (*lane 2*) and hRad51 Gln^{313} (*lane 3*), 3 μg each, were analyzed in an 11% SDS-polyacrylamide gel and stained with Coomassie Blue. *B*, purified hRPA, 3 μg in *lane 2*, was analyzed in a 12.5% SDS-polyacrylamide gel and stained with Coomassie Blue. The three subunits of hRPA are denoted by the *arrows*.

is incorporated last. Pairing between the DNA substrates yields a joint molecule, and branch migration, if successful, over 5.4 kilobase pairs produces nicked circular duplex as product (Fig. 2*A*). We have examined a variety of reaction conditions including the levels of magnesium, pH, and various types of salt on the hRad51 recombinase activity. As documented below, the most dramatic effects were seen with the addition of salts.

We tested the effects of increasing concentrations of potassium acetate, potassium chloride, potassium phosphate, potassium sulfate, ammonium chloride, and ammonium sulfate, and found that while all of these salts were stimulatory, ammonium sulfate produced the most stimulation, followed by potassium sulfate. *Panel I* in Fig. 2*B* shows a time course experiment in which 7.5 μM hRad51 was used with 2 μM hRPA, ϕX (+)-strand (30 μM nucleotides), and ϕX linear duplex (30 μM nucleotides) in pH 7.8 buffer and 100 mM ammonium sulfate. Following the published conditions of Baumann and West (9, 10), another reaction was also carried out in which hRad51, at 5 μM, was used with 1 μM hRPA and the same concentrations of DNA substrates and 80 mM potassium acetate at pH 7.5 (Fig. 2*B, panel II*). The results showed a much higher level of DNA strand exchange under the new conditions. Specifically, whereas no full strand exchange product (nicked circular duplex) was detected under the published conditions (Fig. 2, *B, panel II,* and *C, panel I*) (9, 10), the inclusion of ammonium sulfate resulted in conversion of ~30 and ~60% of the linear duplex to nicked circular duplex after 30 and 60 min, respectively (Fig. 2, *B, panel I,* and *C, panel I*). Overall, there was a 3–4-fold increase in total products (joint molecules plus nicked circular duplex) in the reaction that employed ammonium sulfate (Fig. 2*C, panel II*). Even though 100 mM ammonium sulfate was found to be optimal, highly significant levels of homologous DNA pairing and complete DNA strand exchange were seen at reduced concentrations (50 and 75 mM) of the salt (data not shown).

In published studies (9, 10), 80 mM potassium acetate was employed. In agreement with the published work (9, 10), neither higher (up to 200 mM in 20 mM increments) nor lower concentrations of potassium acetate would improve homologous DNA pairing and strand exchange efficiency beyond the level seen in *panel II* of Fig. 2*B*. As expected from published work (8), omission of ATP from the reaction abolished strand exchange, either under our reaction conditions (Fig. 2*B, panel I, lanes 10–12*) or the published conditions (Fig. 2*B, panel II, lanes 11–13*) (9, 10).

Thus, the inclusion of ammonium sulfate renders hRad51-mediated ATP-dependent homologous DNA pairing and strand exchange highly efficient. Under the new reaction conditions, the optimal levels of hRad51 for pairing and strand exchange were found to be between 2 and 4 nucleotides/protein monomer. Increasing hRad51 above 2 nucleotides/protein monomer resulted in gradual inhibition (data not shown), which was likely due to binding of hRad51 to the duplex and its sequestration from pairing with the hRad51-ssDNA complex (7, 9).

Dependence on hRPA—In the yRad51-mediated DNA strand exchange reaction that uses plasmid length DNA substrates, a strong dependence of the reaction efficiency on yRPA has been observed (2, 4). However, in the published work, when hRad51 was used at the optimal ratio of 3 nucleotides of ssDNA/hRad51 monomer, hRPA has no stimulatory effect on the reaction efficiency, and relatively high levels of hRPA were in fact strongly inhibitory (9, 10). We have examined whether under the newly devised reaction conditions, hRPA is required for strand exchange efficiency. Fig. 3 summarizes the results obtained with 7.5 μM hRad51, 30 μM nucleotides of ssDNA, 100 mM ammonium sulfate, and increasing concentrations of hRPA, from 0.4 to 4.0 μM. Whereas only negligible pairing and strand exchange was seen in the absence of hRPA, increasing concentrations of hRPA gave progressively higher levels of products (Fig. 3, panels I and *II*). The optimal level of hRPA was ~2 μM, although addition of as little as 0.4 μM hRPA gave highly notable stimulation. Importantly, increasing the hRPA concentration to 4 μM did not lower the level of products, which is very different from published studies (10) in which concentrations of hRPA $\geq$0.8 μM were found to be strongly inhibitory.

Additional experiments revealed that at levels of hRad51 higher (2 nucleotides/hRad51 monomer) or lower (6 nucleotides/hRad51 monomer) than that (4 nucleotides/hRad51 monomer) used in Fig. 3, there is also a similar dependence of homologous pairing and strand exchange on hRPA. Likewise, at ammonium sulfate levels higher and lower than that used in prior experiments, we have also observed a similar dependence of the strand exchange reaction on hRPA. Control experiments confirmed that hRPA by itself does not have homologous pairing and strand exchange activity under the new conditions (data not shown). In summary, under our reaction conditions, there is a uniform dependence of DNA strand exchange on hRPA, regardless of the amount of hRad51 used.

Effect of Order of Addition of Salt and Heterologous DNA—In the experiments described thus far, ammonium sulfate was added to the reaction mixture after hRad51 had already nucleated onto the ssDNA but before the incorporation of hRPA. We have also examined whether the addition of ammonium sulfate at other stages would affect the reaction efficiency, as such an endeavor could yield important clues as to the basis of stimulation. As shown in Fig. 4, similar levels of homologous DNA pairing and strand exchange were observed when ammonium sulfate was added at the beginning with hRad51, after hRad51 but before the incorporation of hRPA (as in the standard reaction), and after hRPA but before the incorporation of the duplex. Interestingly, when ammonium sulfate was incorporated a few minutes after the duplex, there was little product formed even at the reaction end point of 60 min (Fig. 4*A, lanes 11–13*). Since dsDNA coated with hRad51 or yRad51 has been found to be inactive in the DNA strand exchange reaction (7, 9), we considered the possibility that perhaps the suppression of DNA strand exchange seen with ammonium sulfate being added after the duplex might have stemmed from free hRad51 binding to the duplex (7). However, two lines of evidence strongly suggest that this was not the main reason for the lack of strand exchange stimulation. First, even at levels of hRad51 (6 nucleotides and 9 nucleotides of ssDNA/hRad51 monomer) lower than that (4 nucleotides ssDNA/hRad51 monomer) used in Fig.

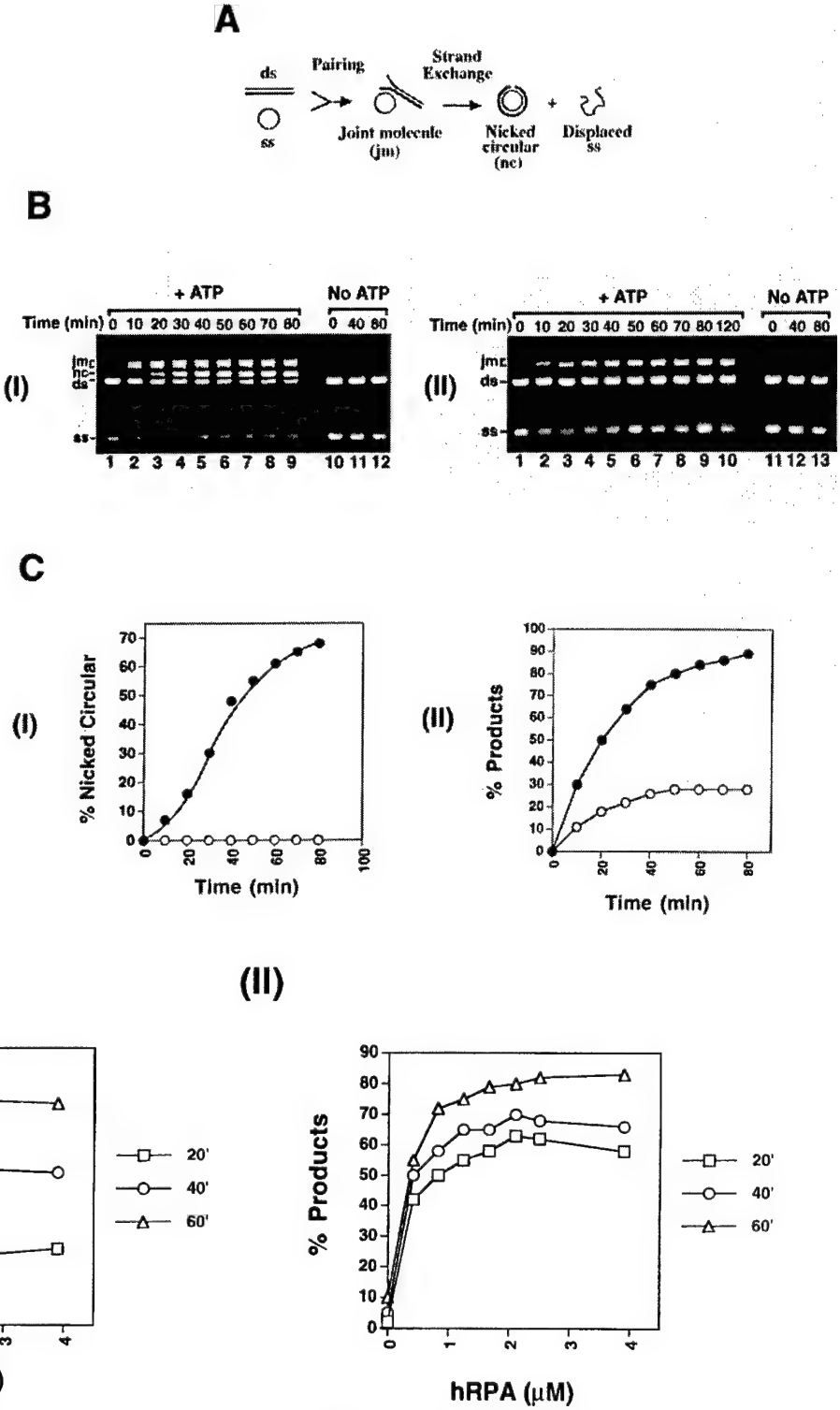

FIG. 2. **Homologous DNA pairing and strand exchange by hRad51 and hRPA.** *A*, schematic of the reaction. The ϕX174 viral (+)-strand (*ss*) is paired with the linear homologous duplex (*ds*) to form joint molecules (*jm*), and strand exchange over the length of the DNA (5.4 kilobase pairs) yields nicked circular duplex (*nc*) and linear ssDNA as products. *B*, hRad51-mediated homologous DNA pairing and strand exchange. *Panel I* shows reactions carried out with ammonium sulfate as salt and with ATP (*lanes 1–9*) or without ATP (*lanes 10–12*). The reactant concentrations were: 7.5 μM hRad51, 2 μM hRPA, 30 μM (in nucleotides) each of ssDNA and dsDNA. *Panel II* shows reactions carried out as described by Baumann and West (9, 10), with ATP (*lanes 1–10*) or without it (*lanes 11–13*). The reactant concentrations were: 5 μM hRad51, 1 μM hRPA, and 30 μM (in nucleotides) each of ssDNA and dsDNA. *C*, in *panel I*, the time courses of conversion of the input linear duplex into nicked circular duplex in the experiments in *panels I* (●) and *II* (○) of *B* are graphed. In *panel II*, the time courses of conversion of the input linear duplex into total products (joint molecules and nicked circular duplex) in the experiments in *panels I* (●) and *II* (○) of *B* are graphed.

FIG. 3. **Dependence of homologous DNA pairing and strand exchange on hRPA.** hRad51 at 7.5 μM was incubated with 30 μM ϕX ssDNA with or without increasing concentrations of hRPA, and the resulting hRad51-ssDNA nucleoprotein filaments were reacted with linear ϕX dsDNA for 20 (□), 40 (○), and 60 (△) min. In *panel I*, the level of full DNA strand exchange, as measured by the percent conversion of the input linear duplex to nicked circular duplex, was graphed. In *panel II*, the level of total products, joint molecules plus nicked circular duplex, was graphed.

4 and with much longer preincubation of hRad51 with ssDNA to minimize the level of free hRad51, addition of ammonium sulfate before the duplex molecule is still necessary to see significant strand exchange (data not shown). Second, an excess of a heterologous duplex (pBluescript) added together with the homologous duplex or before the homologous duplex, in an attempt to titrate out any free hRad51, also did not compensate for the stimulatory effect of adding ammonium sulfate before the homologous duplex (see below). Taken together, the results strongly suggested that the lack of DNA strand exchange when ammonium sulfate was incorporated after the homologous duplex was due to a reason other than free hRad51 coating the duplex molecule.

Interestingly, whereas the incorporation of increasing

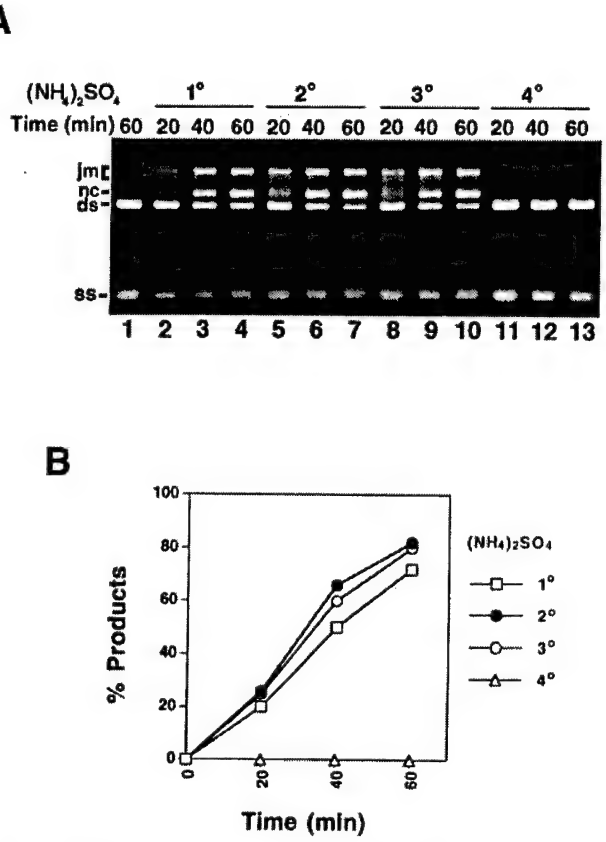

FIG. 4. **Order of addition of salt is critical for reaction efficiency.** *A*, ammonium sulfate (100 mM) was added at the same time as hRad51 to ϕX ssDNA (1°), after ϕX ssDNA had been preincubated with hRad51 (2°), after ϕX ssDNA had been preincubated first with hRad51 and then with hRPA (3°), or added 3 min after the duplex had already been incorporated into the reaction (4°). The complete reaction mixtures were incubated for 20, 40, and 60 min and processed for gel analysis. In *lane 1*, DNA substrates were incubated in the absence of recombination proteins. The reactant concentrations were: hRad51 at 7.5 μM, ϕX ssDNA at 30 μM nucleotides, hRPA at 2 μM, and ϕX dsDNA at 30 μM nucleotides. *B*, the levels of total products, joint molecules plus nicked circular duplex, were graphed.

amounts of the heterologous pBluescript duplex in the presence of ammonium sulfate lowered the reaction efficiency only slightly (Fig. 5, *A, panel I*, and *B, panel I*), the addition of pBluescript duplex before ammonium sulfate resulted in much more pronounced inhibition (Fig. 5, *A, panel II*, and *B, panel II*). These results, coupled with those presented above, indicated that binding of duplex to the hRad51-ssDNA nucleoprotein filament, regardless of whether the duplex is homologous to the ssDNA situated in the hRad51 filament, has a strong suppressive effect on pairing and strand exchange, unless salt is already present.

In summary, the results have revealed a strict dependence of homologous DNA pairing and strand exchange efficiency on ammonium sulfate being incorporated into the reaction prior to the duplex, and they suggest that ammonium sulfate exerts its stimulatory effect via modulation of the interactions between the hRad51-ssDNA nucleoprotein complex and the incoming duplex molecule. This premise is further tested and verified in the experiments below.

Dependence of Strand Exchange Efficiency on Interactions between Duplex and hRad51-ssDNA Complex—Extensive biochemical studies conducted with RecA have revealed that the incoming duplex molecule is bound only transiently within the RecA-ssDNA nucleoprotein filament (4, 5, see "Discussion"). We reasoned that if the hRad51-ssDNA filament has a relatively high affinity for the duplex, then the DNA homology search process might occur efficiently only when the association of the duplex molecule and the hRad51-ssDNA nucleoprotein filament is rendered transient, which could conceivably be realized by salt inclusion.

Intrinsic to this hypothesis are two predictions. First, it might be expected that the salt dependence of the homologous DNA pairing and strand exchange process would be lessened with reduction in the length of the DNA substrates, such as when oligonucleotides are used (11) (see Fig. 6*A* for schematic). This is because the extent of interactions of a short duplex with the limited length of hRad51-ssDNA nucleoprotein filament assembled on a short single-strand would not be extensive. Furthermore, the search for DNA homology with short DNA substrates would not be as rate-limiting as when ϕX DNA substrates are used, because the probability of productive collisions between two short substrates leading to their homologous registry should be considerably higher. As predicted, when the DNA substrates used were based on 83-mer oligonucleotides, the rate of homologous pairing between the substrates was the same in the absence of salt as when ammonium sulfate was present at 25 mM, and higher levels of ammonium sulfate were in fact inhibitory (Fig. 6, *B* and *C*).

Second, if salt indeed acted to weaken the interaction between the duplex and the hRad51-ssDNA nucleoprotein complex, then we would expect to see lessened binding of the duplex molecule to the hRad51-ssDNA complex when ammonium sulfate was present. To test this premise experimentally, we examined the interaction of ϕX linear dsDNA with hRad51-ϕX ssDNA complex immobilized on streptavidin magnetic beads via a short biotinylated oligonucleotide, called F1b, which is complementary to a portion of the ϕX (+)-strand (see schematic in *panel I* of Fig. 7*A* and "Experimental Procedures"). Analysis of the SDS eluate of the magnetic beads allowed us to determine the amount of duplex DNA that had bound to the immobilized hRad51-ssDNA complex (Fig. 7*A*, *panel I*). As shown in Fig. 7*A*, *lanes 1* and *2* in *panel II*, incubation of the duplex with bead-immobilized hRad51-ssDNA complex in the absence of ammonium sulfate resulted in >90% retention of the duplex on the beads. Binding of the duplex to the magnetic beads was due to its interaction with the immobilized hRad51-ssDNA complex, because little retention of the duplex DNA occurred with magnetic beads pretreated with hRad51 and ϕX (+)-strand hybridized to an oligonucleotide, F1, that had identical sequence to F1b but lacked the biotin tag of the latter (Fig. 7*A*, *lanes 3* and *4* in *panel II*). As expected, only the background level of duplex retention was seen with beads containing the F1b-ϕX (+)-strand hybrid but without hRad51, with DNA-free beads preincubated with hRad51, and with beads that contained only the F1b oligonucleotide and preincubated with hRad51 (Fig. 7*A*, *lanes 5–10* in *panel II*).

Once the utility of the assay system was verified, we proceeded to test the effect of ammonium sulfate on the interactions of duplex DNA with the immobilized hRad51-ssDNA complex. The results revealed gradual weakening of the duplex/hRad51-ssDNA complex interactions by increasing levels of ammonium sulfate. Specifically, whereas greater than 90% retention of the duplex occurred in the absence of ammonium sulfate, less than 10% of the duplex was bound at 100 mM of the salt (Fig. 7, *B, upper panel*, and *C*). Analysis of the amount of hRad51 in the various SDS eluates showed that even the highest concentration of ammonium sulfate did not cause significant turnover of hRad51 from the bound ssDNA (Fig. 7*B, lower panel*). Taken together, we concluded that ammonium sulfate indeed weakens the binding of duplex DNA to the hRad51-

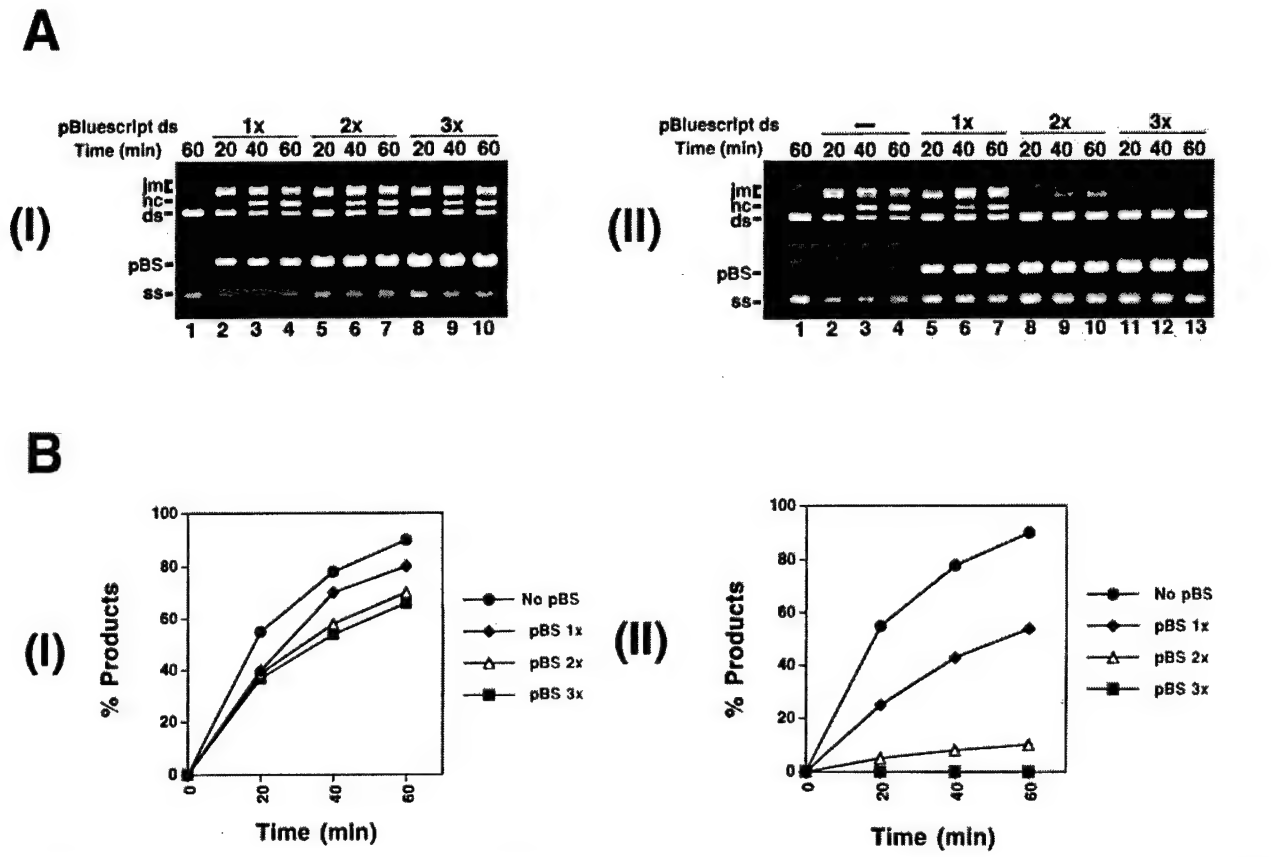

FIG. 5. **Effect of heterologous duplex.** *A,* increasing concentrations of pBluescript (1 to 3 times the concentration of ϕX dsDNA) was added in the presence of ammonium sulfate (*panel I*) or 3 min before the incorporation of ammonium sulfate (*panel II*) to presynaptic complex assembled with hRad51 (7.5 μM), hRPA (2 μM), and ϕX ssDNA (30 μM nucleotides). Following the incorporation of ϕX duplex (30 μM nucleotides), the reaction mixtures were incubated for the indicated times. The concentration of ammonium sulfate was 100 mM. In *lane 1* of both panels, DNA substrates were incubated in the absence of recombination proteins. *B,* the levels of total products, joint molecules plus nicked circular duplex, in *A* were graphed. *Panel I* shows the levels of products (joint molecules and nicked circular duplex) when pBluescript was added after ammonium sulfate and *panel II* shows the levels of products when pBluescript was added before ammonium sulfate.

ssDNA complex. Other experiments revealed that pBluescript duplex also binds to the immobilized hRad51-ϕX ssDNA complex in a manner that is reduced by ammonium sulfate (data not shown), indicating that the hRad51-ssDNA complex can interact with both homologous and heterologous duplex molecules.

Interestingly, potassium acetate lessened the interaction between the duplex and the immobilized hRad51-ssDNA complex only slightly (Fig. 7*C*), and as expected, the hRad51-ssDNA complex was stable to potassium acetate (data not shown). Since potassium acetate is much less effective in the homologous pairing and strand exchange reaction (Fig. 2) (9, 10), the observation in Fig. 7*C* is again consistent with the suggestion that ammonium sulfate stimulates homologous pairing and strand exchange by attenuating the affinity of the hRad51-ssDNA nucleoprotein filament for the incoming duplex.

In the DNA strand exchange experiments, we found that the order of addition of ammonium sulfate relative to duplex DNA was important for ensuring strand exchange efficiency, such that if duplex DNA was added before ammonium sulfate, only negligible pairing and strand exchange was observed (see Fig. 4). Given this observation, we wanted to test whether the level of duplex retention by bead-immobilized hRad51-ssDNA complex would change with the order of addition of ammonium sulfate. To examine this, we used three different concentrations of ammonium sulfate (25, 50, and 100 mM) and added the salt either before the incorporation of duplex or after the duplex had already been preincubated with the bead-immobilized hRad51-ssDNA complex. The results from this experiment revealed that more of the duplex becomes associated with the hRad51-ssDNA complex with preincubation of duplex and Rad51-ssDNA complex prior to salt addition (Fig. 7*D*).

The experiments in Fig. 7 were conducted with hRad51-ssDNA nucleoprotein complex assembled in the absence of hRPA. We have obtained similar results when hRPA was included in the binding reaction (data not shown).

DISCUSSION

Homologous DNA Pairing and Strand Exchange by hRad51 and hRPA—Both hRad51 and yRad51 are related in amino acid sequence and biological function to *E. coli* RecA. Like RecA, yRad51 forms nucleoprotein filaments on ssDNA and dsDNA in an ATP-dependent manner. Biochemical studies have indicated that the search for DNA homology in the incoming duplex DNA molecule and formation of heteroduplex joints with the duplex occur within the confines of the RecA-ssDNA and yRad51-ssDNA nucleoprotein filaments, which are also referred to presynaptic filaments. The assembly of the recombinase-ssDNA nucleoprotein filaments and the efficiency of subsequent homologous pairing and strand exchange are stimulated by the single-strand binding factor, SSB for RecA and yRPA for yRad51 (2, 4, 5). In the presence of ATP, hRad51 also forms a filament on ssDNA similar in structure to the equivalent nucleoprotein filaments assembled with RecA and yRad51 (3–5). However, published studies have suggested that hRad51 has only a modest ability to make DNA joints and an even lower capacity to promote DNA strand exchange. These published observations have suggested that either hRad51 partic-

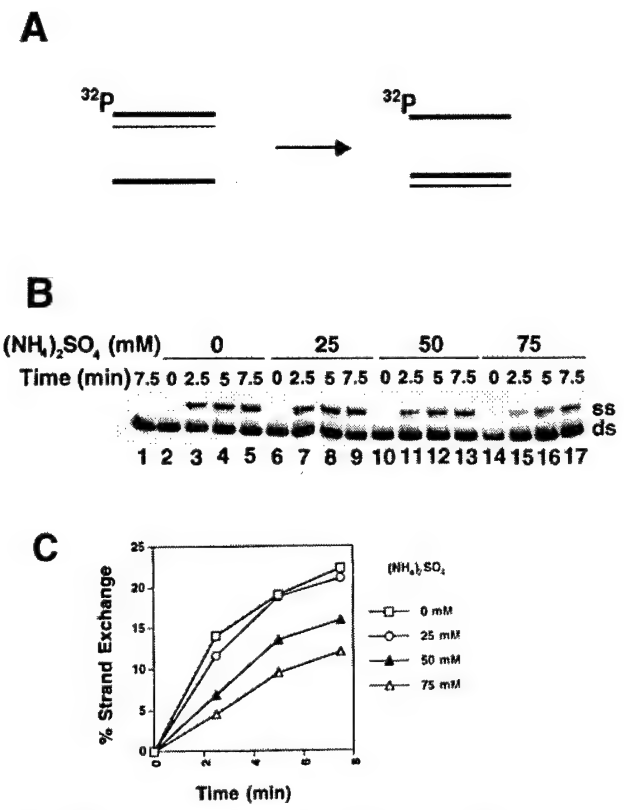

FIG. 6. **Homologous pairing with oligonucleotide-based substrates is independent of salt.** *A*, pairing and strand exchange between the unlabeled single-stranded oligonucleotide with the ^{32}P-labeled duplex results in displacement of the radiolabeled single strand. *B*, hRad51 was incubated with the 83-mer oligonucleotide and the resulting nucleoprotein filament was reacted with the homologous duplex in the absence of salt or with increasing concentrations of ammonium sulfate, as indicated. In *lane 1*, DNA substrates were incubated in the absence of hRad51. *C*, the results from PhosphorImager analysis of the gel in *B* were plotted.

ipates in making DNA joints without catalyzing much DNA strand exchange, as discussed before (3), or that other reaction conditions are in fact required to reveal the strand exchange activity in hRad51. To entertain the latter possibility, we have explored a variety of reaction parameters for their effect on the hRad51 recombinase activity, and have shown that when ammonium sulfate is included, an avid capability of hRad51 to catalyze DNA joint formation and strand exchange is revealed. Moreover, formation of nicked circular duplex, the product of full DNA strand exchange, becomes completely dependent on hRPA. The dependence of homologous DNA pairing and strand exchange on hRPA is seen over a wide range of hRad51 concentrations, from below and above the optimal level. The requirement for hRPA in homologous DNA pairing and strand exchange is very likely due to its ability to minimize secondary structure in DNA, thus facilitating the assembly of a contiguous hRad51-ssDNA filament, as suggested in previous studies (2–5).

In summary, the results presented here demonstrate an intrinsic ability of hRad51 to form DNA joints efficiently and to catalyze a substantial amount of DNA strand exchange. These findings also reveal the functional dependence of hRad51 recombinase on the ssDNA binding factor hRPA. The ability of hRad51 and hRPA to promote DNA joint formation and extension of nascent heteroduplex joints by strand exchange is likely to be indispensable for various recombination reactions *in vivo*.

Possible Basis for Salt Stimulation of Homologous DNA Pairing and Strand Exchange—Extensive biochemical studies have revealed the presence of two distinct DNA-binding sites in the RecA protein filament, with the initiating ssDNA substrate viewed as being situated within the "primary" site, while the incoming duplex molecule is bound within the "secondary" site of the presynaptic filament (4, 5). The search for DNA homology in the duplex DNA occurs by way of reiterative binding and release of the duplex until homology is located. For this random collision mode of DNA homology search to work efficiently, the incoming duplex molecule must be retained only transiently within the secondary site of the recombinase-ssDNA filament. Consistent with this deduction, evidence has been presented to suggest that the RecA-ssDNA filament has modest affinity for duplex DNA (4).

Given the structural and functional similarities between hRad51 and RecA, it seems reasonable to suggest that DNA homology conducted by the hRad51-ssDNA presynaptic filament also occurs by means of random association/dissociation of the duplex molecule with the former, with efficient homology search to be dictated by transient, rather than stable, association of the incoming duplex with the presynaptic filament. We have presented two lines of evidence to support the notion that salt exerts its remarkable stimulatory effect by weakening the interactions of the duplex molecule with the hRad51-ssDNA filament, thereby enhancing the rate of turnover and the efficiency at which the duplex can be sampled for homology. First, we have shown that the dependence of the homologous DNA pairing and strand exchange process on salt is alleviated if the length of the DNA substrates is reduced, as when oligonucleotides are used. This is likely due to the increased probability for productive collisions between the two oligonucleotide substrates that lead to homologous registry and also because the interactions between the duplex and the short presynaptic filament of hRad51 assembled on the ss oligonucleotide are similarly minimized. Furthermore, we have provided direct evidence that binding of a duplex molecule to hRad51 presynaptic filament is weakened by the inclusion of ammonium sulfate.

In summary, we surmise from the biochemical results that the hRad51-ssDNA filament binds duplex DNA in the available secondary site with high affinity, thus limiting efficient sampling of the duplex molecule for DNA homology. Accordingly, stimulation of homologous DNA pairing is realized by salt addition, with the degree of stimulation being dependent on the effectiveness of a particular salt to weaken the affinity of the hRad51-ssDNA nucleoprotein filament for the duplex molecule. We further suggest that the stimulation of strand exchange efficiency by ammonium sulfate is also due to increased turnover of the duplex DNA from the secondary DNA-binding site, as the ease with which the initial DNA joint can be extended by branch migration may be expected to be critically dependent on the hRad51-ssDNA nucleoprotein filament being free of stably bound duplex DNA molecules as well. These suggestions are summarized in our working model in Fig. 8.

It is reasonable to ask whether the requirement for a high level of salt for revealing the catalytic potential of the hRad51 recombinase is physiological. Concerning this point, it is important to note that the *in vivo* salt concentration is between 0.17 and 0.24 M (17). The specific stimulation of hRad51 strand exchange activity by salts could also be reflecting the requirement for a small molecule (a polyanion, for instance), a certain post-translational modification of hRad51 (phosphorylation, for instance), or the involvement of other recombination factors (see discussion below) in modulating the affinity of the hRad51-ssDNA filament for the incoming duplex DNA molecule.

Significance of the in Vitro DNA Strand Exchange System—Studies on yRad51 using plasmid length DNA molecules as

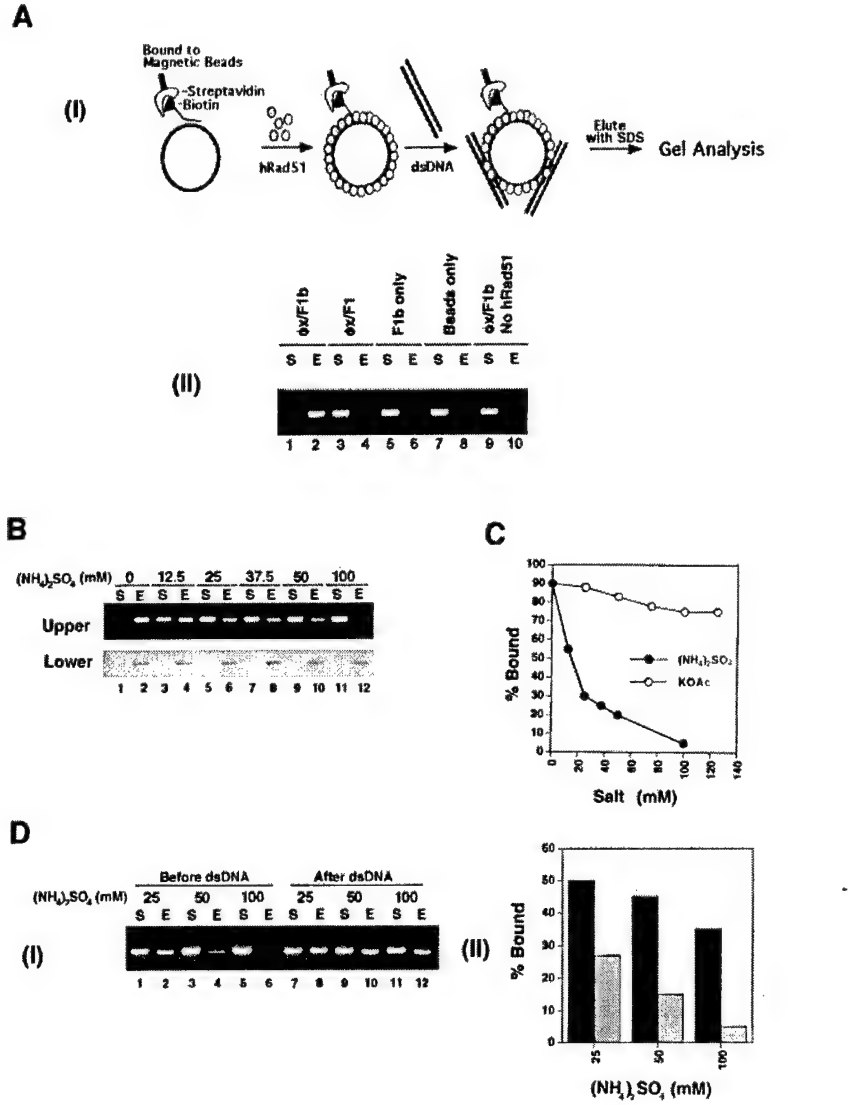

FIG. 7. **Salt lessens interactions between duplex and hRad51-ssDNA nucleoprotein filament.** *A, panel I,* the assay scheme is summarized. Briefly, ϕX (+)-strand hybridized to the biotinylated oligonucleotide F1b is immobilized on streptavidin magnetic beads via the biotin tag. hRad51 filament is assembled on the (+)-strand and then mixed with ϕX duplex DNA. The bound duplex and hRad51 are eluted by SDS and analyzed. To verify the utility of the assay (*panel II*), beads containing ϕX (+)-strand-F1b hybrid (*ϕX-F1b*; *lanes 1* and *2*), beads preincubated with ϕX (+)-strand hybridized to the nonbiotinylated oligonucleotide F1 (*ϕX-F1*; *lanes 3* and *4*), beads with F1b but no ϕX (+)-strand (*F1b only*; *lanes 5* and *6*), and beads that contained neither ϕX (+)-strand nor F1b (*beads only; lanes 7* and *8*) were incubated with hRad51 and then mixed with ϕX duplex. As an additional control, ϕX duplex DNA was incubated with beads containing the ϕX (+)-strand-F1b hybrid without hRad51 (*ϕX/F1b, No hRad51; lanes 9* and *10*). The supernatants (*S*) and the SDS eluates (*E*) from the reactions were analyzed in an agarose gel for their content of duplex DNA. *B,* salt weakens interaction of duplex with the hRad51-ssDNA filament. Duplex ϕX DNA was incubated with the hRad51 filament assembled on the immobilized ϕX (+)-strand with increasing concentrations of ammonium sulfate. The supernatants (*S*) and SDS eluates (*E*) from the binding reactions were analyzed for their contents of DNA duplex (*upper panel*) and hRad51 (*lower panel*). *C,* the results in *B* are graphed (●), as are results from binding reactions in which potassium acetate was used (○). *D,* effect of order of addition of ammonium sulfate. Duplex DNA was added to binding reactions 3 min before or immediately after the incorporation of increasing levels of ammonium sulfate (*panel I*). The results with adding ammonium sulfate before (*shaded bar*) and after (*dark bar*) the incorporation of duplex are presented in the histogram in *panel II*.

substrates have been instrumental for formulating biochemical models for understanding the functions of various *RAD52* group proteins. For instance, in addition to the well documented stimulatory role of yRPA in yRad51-mediated DNA strand exchange, experiments which varied the order of addition of reaction components have revealed that yRPA, if added with or before yRad51 to the ssDNA substrate, can also compete with yRad51 for binding sites on the ssDNA and consequently suppress the assembly of yRad51-ssDNA nucleoprotein filament (2). The yeast *RAD52* encoded product and the heterodimeric molecule of yRad55 and yRad57 proteins, referred to as recombination mediators, promote the assembly of the yRad51-ssDNA filament and help overcome the suppression of DNA strand exchange caused by coaddition of yRPA with yRad51 to the ssDNA substrate or by preincubation of ssDNA with yRPA (2, 18).

We have verified that preincubation of ssDNA with hRad51 before the incorporation of hRPA is in fact critical for homologous pairing and strand exchange efficiency,[2] providing evidence that hRPA competes with hRad51 for binding sites on ssDNA. This observation suggests the existence of specific mediators in the human recombination machinery for promoting hRad51-ssDNA filament assembly when there is the need for hRad51 to compete with other single-strand binding factors for sites on the initiating ssDNA substrate. A possible mediator

[2] S. Sigurdsson and P. Sung, unpublished observations.

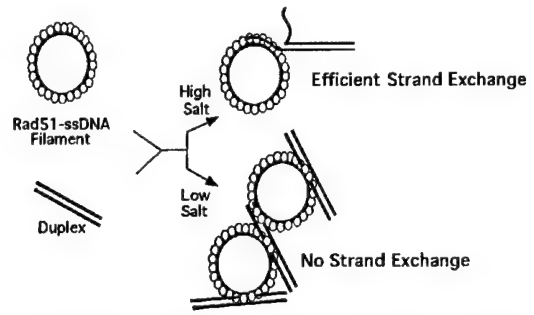

FIG. 8. **Model for hRad51-mediated DNA strand exchange.** The results suggest that duplex DNA molecules stably bound to the hRad51-ssDNA nucleoprotein filament present a strong impediment to the different reaction steps, including DNA homology search, DNA pairing, and branch migration of the nascent DNA joint, that lead to successful recombination between the DNA substrates. Efficient DNA pairing and strand exchange is realized by lessening the duplex/hRad51-ssDNA interactions, achieved by the inclusion of salt.

function may exist in various human recombination factors including hRad52 and a number of Rad55/Rad57-like proteins, namely XRCC2, XRCC3, Rad51B, Rad51C, and Rad51D, which are all known to be involved in recombination and either directly, or through another recombination factor, physically interact with hRad51 (3, 19). Furthermore, it remains a distinct possibility that some of these other recombination factors are in fact integral components of the presynaptic filament, and as such, may modulate the dynamics of the presynaptic filament to facilitate sampling of duplex DNA for homology and to promote the formation of DNA joints once homology is located. The *in vitro* DNA strand exchange system with the defined biochemical parameters described herein should be well suited for examining the function of various recombination factors and the role of post-translational modifications of these recombination factors in the DNA strand exchange reaction.

Recently, hRad51 was shown to interact with the breast tumor suppressor BRCA2 (12). In the Capan-1 cell line defective in BRCA2 function, the DNA damage-induced formation of hRad51 nuclear foci is defective, suggesting the possibility that BRCA2 helps deliver hRad51 to the DNA substrate (12). Whether or not BRCA2 functions as a mediator to promote hRad51 nucleoprotein filament assembly can be tested with the *in vitro* DNA strand exchange system described herein.

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Mediator function of the human Rad51B–Rad51C complex in Rad51/RPA-catalyzed DNA strand exchange

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Five Rad51-like proteins, referred to as Rad51 paralogs, have been described in vertebrates. We show that two of them, Rad51B and Rad51C, are associated in a stable complex. Rad51B–Rad51C complex has ssDNA binding and ssDNA-stimulated ATPase activities. We also examined the functional interaction of Rad51B–Rad51C with Rad51 and RPA. Even though RPA enhances Rad51-catalyzed DNA joint formation via removal of secondary structure in the ssDNA substrate, it can also compete with Rad51 for binding to the substrate, leading to suppressed reaction efficiency. The competition by RPA for substrate binding can be partially alleviated by Rad51B–Rad51C. This recombination mediator function of Rad51B–Rad51C is likely required for the assembly of the Rad51-ssDNA nucleoprotein filament in vivo.

[*Key Words:* DNA double-strand break repair; tumor suppression]

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Studies in *Saccharomyces cerevisiae* have identified a large number of genetic loci required for mitotic and meiotic recombination. These genes, comprising *RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, RDH54/TID1, MRE11,* and *XRS2* are collectively known as the *RAD52* epistasis group. The *RAD52* group of genes are also intimately involved in the repair of DNA double-strand breaks induced by exogenous agents such as ionizing radiation (Paques and Haber 1999; Sung et al. 2000) and for telomere maintenance in the absence of telomerase.

Cloning, genetic, and biochemical studies have indicated that the structure and function of the *RAD52* group genes are highly conserved among eukaryotes, from yeast to humans (Sung et al. 2000; Thompson and Schild 2001). Interestingly, in mammals, the efficiency of recombination and DNA double-strand break repair is contingent upon the integrity of the tumor suppressors BRCA1 and BRCA2 (Dasika et al. 1999; Moynahan et al. 1999, 2001; Thompson and Schild 2001), underscoring the importance for deciphering the mechanistic basis of the recombination machinery.

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In recombination processes that involve the formation of a DNA double-strand break, the ends of the DNA break are processed to yield single-stranded DNA tails. These DNA tails are utilized by the *RAD52* group recombination factors for the formation of DNA joints with a homologous DNA template, contained within the sister chromatid or the chromosomal homolog. The nascent DNA joints are then extended in length by branch migration, followed by resolution of DNA intermediates to complete the recombination process (Paques and Haber 1999; Sung et al. 2000).

The *RAD51* encoded product is the functional homolog of *Escherichia coli* RecA protein, and like RecA, possesses the ability to promote the homologous DNA pairing and strand exchange reaction that forms heteroduplex DNA joints. In mediating homologous DNA pairing and strand exchange, Rad51 must first assemble onto ssDNA as a nucleoprotein filament, in which the DNA is held in a highly extended conformation (Ogawa et al. 1993; Benson et al. 1994; Sung and Robberson 1995). Assembly of the Rad51-ssDNA nucleoprotein filament is rate-limiting and strongly inhibited by secondary structure in the ssDNA template. The removal of secondary structure can be effected by the single-strand DNA binding protein RPA, which has proved to be indispensable for homologous DNA pairing and strand exchange effi-

ciency, especially when a plasmid-length ssDNA template is used as the initiating substrate (Sung et al. 2000; Sigurdsson et al. 2001).

Even though RPA is an important accessory factor for Rad51-mediated homologous DNA pairing and strand exchange, it can also compete with Rad51 for binding sites on the ssDNA template, which, when allowed to occur, suppresses pairing and strand exchange efficiency markedly (Sung et al. 2000). Here we demonstrate that the stoichiometric complex of the human Rad51B and Rad51C proteins, homologs of the *S. cerevisiae* Rad55 and Rad57 proteins (Sung et al. 2000), can partially overcome the suppressive effect of hRPA on hRad51-catalyzed DNA pairing and strand exchange, thus identifying the Rad51B–Rad51C complex as a mediator of recombination.

Results

Rad51B and Rad51C are associated in a stable complex

Because Rad51B and Rad51C interact in two-hybrid studies (Schild et al. 2000), we wished to address whether they are associated in human cells. For detecting Rad51B and Rad51C, we raised antibodies against these proteins expressed in *E. coli* and purified from inclusion bodies by preparative SDS-PAGE. The specificity of the anti-Rad51B and anti-Rad51C antibodies is shown in Figure 1A, in the left and right panels, in which extracts from yeast cells harboring the empty protein expression vector and plasmids expressing Rad51B and Rad51C were probed with the antibodies. A single 40-kD Rad51B and 42-kD Rad51C species was detected. The observed sizes of Rad51B and Rad51C are in good agreement with the predicted values of 39 kD for Rad51B and 42 kD for Rad51C. Furthermore, Rad51B and Rad51C endogenous to human HeLa cells have the same gel sizes as proteins expressed in yeast cells (see below). In both the immunoblot analysis (Fig. 1A) and immunoprecipitation (data not shown), the anti-Rad51B antibodies did not cross-react with Rad51C protein, nor did the anti-Rad51C antibodies cross-react with Rad51B protein.

To identify Rad51B and Rad51C endogenous to human cells, an extract from HeLa cells was fractionated in a Q Sepharose column, and the column fractions were subjected to immunoblotting analysis. In this analysis, we used extracts from yeast cells expressing Rad51B and Rad51C (Fig. 1B, lane 10 in both panels) to aid in the identification of the endogenous proteins. The results (Fig. 1B) showed that Rad51B and Rad51C coeluted from Q Sepharose precisely, from fractions 8 to 16. To investigate whether Rad51B and Rad51C in the Q Sepharose fractions were stably associated, we examined whether they could be coimmunoprecipitated. As shown in Figure 1C, anti-Rad51B antibodies precipitated not only Rad51B, but also Rad51C. Similarly, Rad51B coprecipitated with Rad51C in the anti-Rad51C immunoprecipitation (Fig. 1C). Importantly, the quantity of Rad51B and Rad51C that coprecipitated with the other protein was similar to the amount of these proteins precipitated by their cognate antibodies, suggesting that Rad51B and Rad51C in the Q column fractions were associated as a stable complex. Neither Rad51B nor Rad51C was precipitated by control antibodies raised against yeast Srs2 protein (Fig. 1C). Rad51C also forms a complex with XRCC3 (Schild et al. 2000; Kurumizaka et al. 2001; Mas-

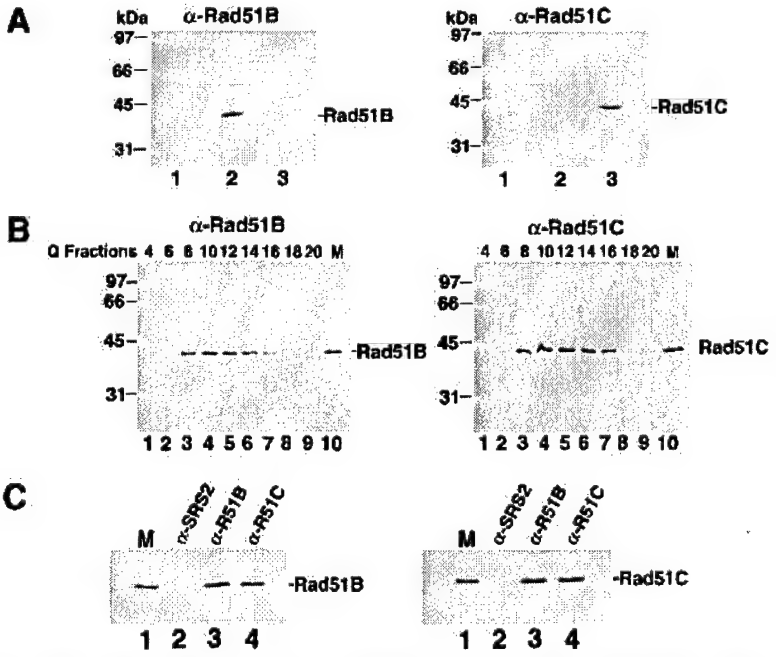

Figure 1. Rad51B and Rad51C form a stable complex. (*A*) Specificity of antibodies. Yeast cells harboring the empty expression vector pPM231 (*2μ, GAL-PGK*; lane *1*), the Rad51B expression plasmid pR51B.1 (*2μ, GAL-PGK-RAD51B*; lane *2*), and the Rad51C expression plasmid pR51C.1 (*2μ, PGK-RAD51C*; lane *3*) were run in an 11% polyacrylamide gel and then subjected to immunoblot analysis with either anti-Rad51B (αRad51B; *left* panel) or anti-Rad51C antibodies (αRad51C; *right* panel). (*B*) Rad51B and Rad51C are associated in a complex in human cells. HeLa cell extract was fractionated in a Q Sepharose column, and the indicated fractions were run in an 11% gel and then subjected to immunoblot analysis with anti-Rad51B antibodies (α-Rad51B; *left* panel) or anti-Rad51C antibodies (α-Rad51C; *right* panel). Yeast extracts containing Rad51B (lane *10* in *left* panel, marked M) or Rad51C (lane *10* in *right* panel, marked M) were used to help identify these proteins in the Q column fractions. (*C*) Coimmunoprecipitation of Rad51B and Rad51C from the Q column fractions. The Q Sepharose pool (fractions 8–16) was subjected to immunoprecipitation with protein A beads containing anti-ySrs2 antibodies (α-SRS2), anti-Rad51B antibodies (α-R51B) and anti-Rad51C antibodies (α-R51C). Proteins bound to the various immunobeads were eluted by SDS treatment and analyzed for their content of Rad51B (*left* panel) and Rad51C (*right* panel).

son et al. 2001), and it remains possible that a portion of Rad51C in the Q Sepharose fractions was bound to XRCC3 or that the XRCC3-Rad51C complex was not retained on the Q column.

The results above indicated that Rad51B and Rad51C are associated in a stable complex in HeLa cell extract, but they could not address whether association of these two proteins was due to direct interaction between them or whether an intermediary is required. To determine whether Rad51B and Rad51C interact directly, we carried out immunoprecipitation using extracts from yeast cells that expressed these two factors. As expected, Rad51B and Rad51C were precipitated by their cognate antibodies but not by antibodies specific for the other protein (data not shown). Importantly, upon mixing of the extracts containing Rad51B and Rad51C, coprecipitation of the two proteins occurred (data not shown). Direct interaction between Rad51B and Rad51C was demonstrated another way. In this case, we constructed recombinant baculoviruses that encoded a six-histidine-tagged form of Rad51B and an untagged form of Rad51C. The expression of Rad51B and Rad51C in insect cells infected separately with these baculoviruses was verified by immunoblotting (Fig. 2A). Because Rad51B was tagged with a six-histidine sequence, we could in this instance use affinity binding of the six-histidine tag to nickel-NTA agarose as criterion for protein–protein interaction when extracts were mixed. As summarized in Figure 2B, untagged Rad51C alone did not bind the nickel matrix, whereas a significant portion of it was retained on the affinity matrix in the presence of six-histidine-tagged Rad51B. Taken together, these results made it clear that Rad51B and Rad51C form a stable complex via direct interaction. The results presented below further indicated that the complex of Rad51B and Rad51C is highly stable, and contains stoichiometric amounts of the two proteins.

Rad51C expressed in insect cells consists of two closely spaced species (Fig. 2A,B), with the top band having the same gel mobility as Rad51C seen in extract from HeLa cells or yeast cells expressing this protein (data not shown). The slower migrating species of the two Rad51C immunoreactive bands is likely full-size Rad51C, whereas the faster migrating form, which represents ~60% of the total Rad51C amount, could be a proteolytic product or the result of an aberrant expression in insect cells.

Expression and purification of the Rad51B–Rad51C complex

We used insect cells as the medium for the purification of the Rad51B–Rad51C complex for the following two reasons: (1) much larger amounts of Rad51B–Rad51C complex can be obtained from insect cells infected with the recombinant baculoviruses than from HeLa cell extract or yeast cells harboring the Rad51B and Rad51C expression plasmids, and (2) the six-histidine tag engineered in the recombinant Rad51B baculovirus allowed

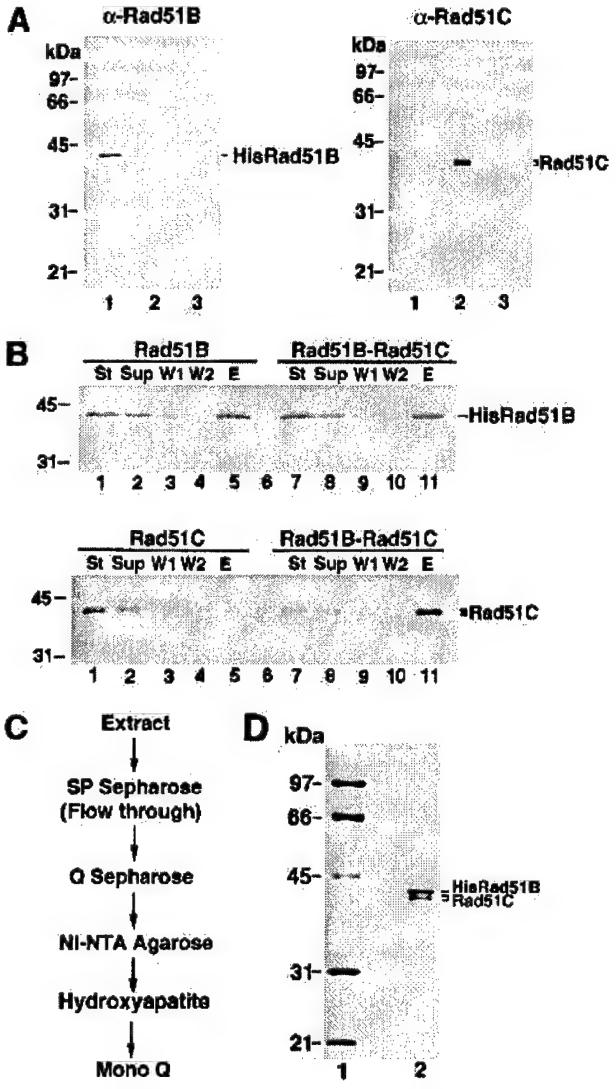

Figure 2. Purification of Rad51B and Rad51C from insect cells. (*A*) Expression of six-histidine-tagged Rad51B and Rad51C in insect cells. Nitrocellulose blots containing extracts from insect cells without any baculovirus (lane 3 of both panels) and infected with the recombinant 6His-tagged Rad51B baculovirus (lane *1* of both panels) or Rad51C baculovirus (lane *2* of both panels) were probed with either anti-Rad51B antibodies (αRad51B; *left* panel) or anti-Rad51C antibodies (αRad51C; *right* panel). (*B*) Complex formation between 6His-tagged Rad51B and Rad51C. Extracts from insect cells expressing 6His-tagged Rad51B (Rad51B), Rad51C (Rad51C), and the mixture of these extracts (Rad51B–Rad51C) were incubated with nickel-NTA agarose beads, which were washed with 10 mM, 20 mM, and then with 150 mM imadazole. The starting fractions (St), the supernatants containing unbound proteins (Sup), the 10 mM (W1) and 20 mM (W2) imidazole washes, and the 150 mM imidazole eluate (E) were subjected to immunoblotting to determine their content of Rad51B (*upper* panel) and Rad51C (*lower* panel). (*C*) Purification scheme for Rad51B–Rad51C complex. (*D*) Purity analysis. The purified Rad51B–Rad51C complex, 1.5 μg in lane 2, was run alongside molecular size markers (lane *1*) in an 11% denaturing polyacrylamide gel and stained with Coomassie blue.

us to use nickel-NTA agarose as an affinity step to facilitate the purification of this complex.

We initially attempted to purify Rad51B and Rad51C individually, but our efforts were hampered by the complications that a significant portion (>75%) of these two proteins was insoluble and the soluble portion gave broad peaks during chromatographic fractionation procedures. To determine whether the Rad51B–Rad51C complex might be more amenable to purification than the individual components, we coinfected the Rad51B and Rad51C recombinant baculoviruses into insect cells. Interestingly, coexpression of Rad51B and Rad51C improved the solubility of these two proteins, even though the overall protein amounts in the infected insect cells remained relatively unchanged (data not shown). Importantly, the Rad51B–Rad51C complex eluted from various chromatographic matrices as relatively well defined peaks, thus enabling us to obtain substantial purification of the complex. Through many small-scale trials, a procedure was devised to encompass fractionation of insect cell extract containing the Rad51B–Rad51C complex in SP Sepharose, Q Sepharose, Hydroxyapatite, Mono Q, and affinity chromatography on nickel-NTA agarose (Fig. 2C) to purify this complex to near homogeneity (Fig. 2D). Stoichiometric amounts of Rad51B and Rad51C cofractionated during the entire purification procedure, indicating a high degree of stability of the complex. Using the aforementioned purification protocol, we could obtain about 100 µg of Rad51B–Rad51C complex from one liter of insect cell culture. Three independent preparations of Rad51B–Rad51C complex gave similar results in the experiments described below.

Rad51B–Rad51C complex binds DNA and hydrolyzes ATP

Because Rad51B and Rad51C are involved in recombination, we tested the purified Rad51B–Rad51C complex for binding to DNA. For this, an increasing amount of Rad51B–Rad51C complex was incubated with either ϕX ssDNA or dsDNA. The reaction mixtures were run in agarose gels, followed by staining with ethidium bromide to detect shifting of the DNA species. As shown in Figure 3A, while clear shifting of the ssDNA occurred at the lowest Rad51B–Rad51C concentration of 0.15 µM (DNA to protein ratio of 80 nucleotides/protein complex; see lane 2 of panel I), no shifting of the dsDNA was seen until the Rad51B–Rad51C concentration reached 0.6 µM (7 base pairs/protein complex; see lane 5 of panel II). These observations suggested that Rad51B–Rad51C complex binds ssDNA readily but has a lower affinity for dsDNA. To validate this conclusion, we repeated the DNA binding experiment by coincubating the ssDNA and dsDNA with the same concentration range of Rad51B–Rad51C used before. The results (Fig. 3A, panel III) revealed that Rad51B–Rad51C complex preferentially shifted the ssDNA without binding significantly to the dsDNA. In these experiments, ATP was included in the buffer used for the binding reaction, but the exclusion of ATP or its substitution with the nonhydrolyzable ATP-γ-S did not affect the binding results appreciably (data not shown). Taken together, these findings led us to conclude that the Rad51B–Rad51C complex binds ssDNA preferentially. We also examined ssDNA binding by Rad51B–Rad51C as a function of the ionic strength. To

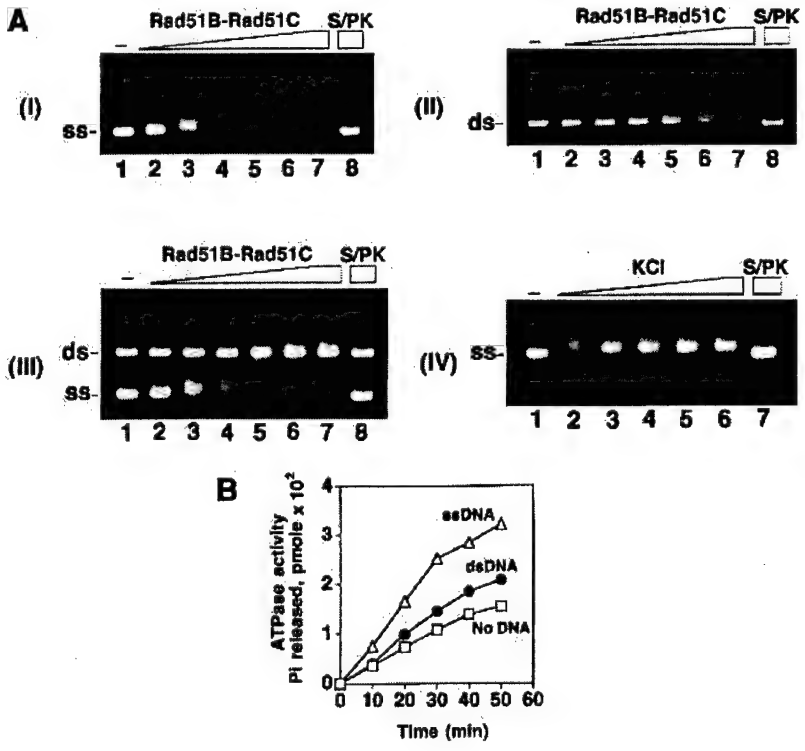

Figure 3. Rad51B–Rad51C binds DNA and hydrolyzes ATP. (*A*) Rad51B–Rad51C complex (0.15, 0.3, 0.45, 0.6, 0.75, and 0.9 µM in lanes *2–7*, respectively) was incubated with ϕX ssDNA (12 µM nucleotides in panel *I*; designated as ss), ϕX dsDNA (4 µM base pair in panel *II*; designated as ds), or with both the ssDNA and dsDNA (panel *III*) for 10 min at 37°C and then run in a 0.9% agarose gel. The DNA species were stained with ethidium bromide. In lane *8* of all three panels, the nucleoprotein complex formed with 0.9 µM of Rad51B–Rad51C complex was treated with 0.5% SDS and 500 µg/mL proteinase K at 37°C for 5 min before loading onto the agarose gel. In lane *1* of all three panels, DNA was incubated in buffer without protein. In panel *IV*, Rad51B–Rad51C complex (0.3 µM) was incubated with ssDNA (12 µM nucleotides) in the presence of increasing concentrations (50, 100, 150, 200, and 250 mM in lanes *2–6*, respectively) of KCl at 37°C for 10 min and then analyzed. (*B*) Rad51B–Rad51C, 1.8 µM, was incubated with 1 mM ATP in the absence of DNA (designated by the squares) and in the presence of ssDNA (20 µM nucleotides; designated by the triangles) or dsDNA (20 µM base pairs; designated by the closed circles) for the indicated times at 37°C.

do this, a fixed quantity of Rad51B–Rad51C complex (0.3 μM) was incubated with the ssDNA (12 μM nucleotides) in the presence of increasing concentrations (50, 100, 150, 200, and 250 mM) of KCl. The results (Fig. 3A, panel IV) showed that ssDNA binding was not diminished significantly by even the highest concentration of KCl (250 mM), indicating a high degree of avidity of Rad51B–Rad51C complex for the DNA.

Both Rad51B and Rad51C contain Walker ATP binding motifs, suggestive of an ability to bind and hydrolyze ATP (Sung et al. 2000; Thompson and Schild 2001). For this reason, we examined the purified Rad51B–Rad51C complex for ATPase activity. As shown in Figure 3B, Rad51B–Rad51C complex possesses an ATP hydrolytic activity that is stimulated by DNA. Reproducibly, ssDNA was more effective at stimulating ATP hydrolysis than was dsDNA. The kcat values for the Rad51B–Rad51C ATPase were found to be 0.15/min in the absence of DNA, and 0.19/min and 0.3/min in the presence of dsDNA and ssDNA, respectively.

Effects of RPA on Rad51-mediated DNA joint formation

Human Rad51 (hRad51) can make joints between homologous single-stranded and double-stranded DNA molecules, but was thought to have only limited DNA strand exchange activity (Baumann and West 1997, 1999; Gupta et al. 1997). We recently described a DNA strand exchange system (Fig. 4A) wherein hRad51 mediates a substantial amount of DNA strand exchange (Sigurdsson et al. 2001). In this new system, the efficiency of homologous DNA pairing and strand exchange is strongly dependent on the heterotrimeric ssDNA binding factor RPA. To assemble the reaction, hRad51 is preincubated with circular ssDNA at the optimal ratio of 4 nucleotides per protein monomer, followed by the addition of

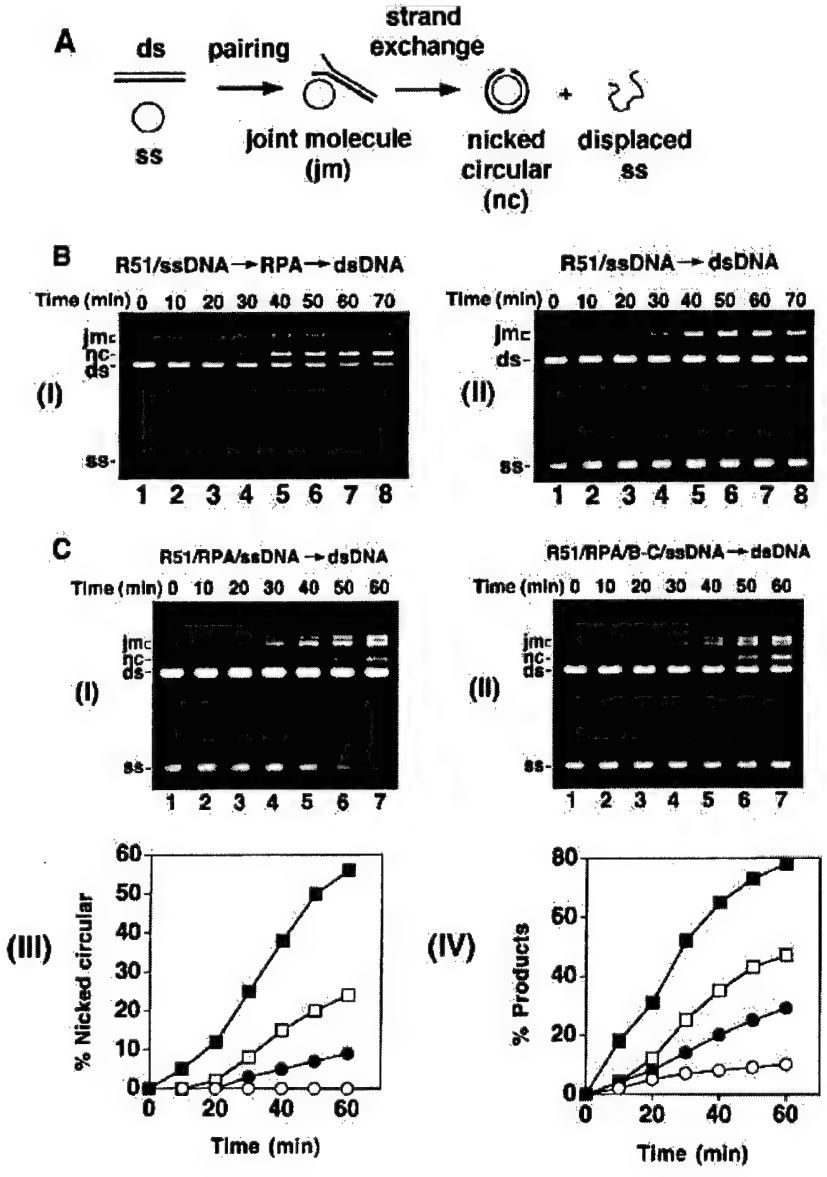

Figure 4. Mediator function of Rad51B–Rad51C. (*A*) Schematic of the homologous DNA pairing and strand exchange reaction using ϕX174 DNA substrates. Linear duplex is paired with the homologous ssDNA circle to yield a joint molecule. DNA strand exchange, if successful over the length (5.4 kb) of the DNA molecules, results in the formation of the nicked circular duplex. (*B*) Rad51-mediated DNA pairing and strand exchange was carried out with RPA (panel *I*) or without it (panel *II*). In panel *I*, the ssDNA was preincubated with Rad51 (R51) before RPA was added. The concentrations of the reaction components were: Rad51, 7.5 μM; RPA, 1.5 μM; ssDNA, 30 μM nucleotides; linear duplex, 15 μM base pairs. (*C*) In the DNA strand exchange reaction in panel *I*, the ssDNA was incubated with both Rad51 (R51) and RPA simultaneously, and in the reaction in panel *II*, the ssDNA was incubated with Rad51, RPA and Rad51B–Rad51C (*B–C*) simultaneously. The concentration of Rad51B–Rad51C was 0.8 μM, while the concentrations of the other components were exactly as those in *B*. In panel *III*, the amounts of nicked circular duplex in the reactions represented in *B* panel *I* (filled squares) and panel *II* (open circles) and in *C* panel *I* (filled circles) and panel *II* (open squares) are plotted. In panel *IV*, the amounts of total reaction products (sum of joint molecules and nicked circular duplex) in the reactions represented in *B* panel *I* (filled squares) and panel *II* (open circles) and in *C* panel *I* (filled circles) and panel *II* (open squares) are plotted.

hRPA at 20 nucleotides per protein monomer and the homologous linear duplex substrate (Fig. 4A). The reaction products, that is, joint molecules and nicked circular duplex (Fig. 4A), are separated from the input substrates by agarose gel electrophoresis and visualized by staining with ethidium bromide. An example of this reaction is shown in Figure 4B, panel I. As reported earlier (Sigurdsson et al. 2001) and reiterated here, omission of RPA from the reaction reduced the amount of products markedly (Fig. 4B, panel II). Importantly, little or no nicked circular duplex, the product of full strand exchange, was generated in the reaction that did not contain RPA (Fig. 4B, panel II).

Interestingly, coaddition of RPA with Rad51 to the ssDNA to mimic what may be expected to occur in vivo resulted in a marked reduction in reaction efficiency (Fig. 4C, panels I, III, and IV). Specifically, while ~38% and ~56% of the input linear duplex substrate had been converted into nicked circular duplex after 40 min and 60 min in the standard reaction (Fig. 4B, panel I and 4C, panel III), coaddition of hRPA with hRad51 to the ssDNA yielded only 5% and 9% of nicked circular duplex after these reaction time (Fig. 4C, panels I and III). In the homologous DNA pairing and strand exchange reaction mediated by *E. coli* RecA or yeast Rad51, SSB/yRPA added to the ssDNA template before or at the same time as the recombinase causes a notable suppression of the reaction as well (Umezu et al. 1993; Sung 1997a; New et al. 1998; Shinohara and Ogawa 1998). In these cases, suppression of the reaction efficiency by SSB/yRPA is due to competition of these ssDNA binding factors with RecA/yRad51 for sites on the ssDNA template. Based on the paradigm established with the *E. coli* and yeast recombination systems (Bianco et al. 1998; Sung et al. 2000), we also attribute the suppression of hRad51-mediated DNA pairing and strand exchange by hRPA to the exclusion of hRad51 from the ssDNA template.

Rad51B–Rad51C complex has a presynaptic mediator function

Rad51B and Rad51C are both required for recombination and DNA double-strand break repair in vivo. In chicken DT40 cells deleted for either Rad51B or Rad51C, the assembly of Rad51 nuclear foci in response to DNA damage is compromised (Takata et al. 2000, 2001). Based on these results, we wished to test whether Rad51B–Rad51C could promote homologous DNA pairing and strand exchange by Rad51 with RPA competing for binding sites on the initiating ssDNA substrate. To do this, we added Rad51B–Rad51C complex (0.8 μM) with Rad51 (7.5 μM) and RPA (1.5 μM) during the preincubation with ssDNA (30 μM), and then completed the reaction mixture by adding the homologous duplex. Importantly, upon inclusion of the Rad51B–Rad51C complex, significantly higher amounts of the reaction products were seen (Fig. 4C, panels II, III, and IV). In particular, the level of the nicked circular duplex, product of complete strand exchange (see Fig. 4A), was formed at a significantly higher rate than in the absence of Rad51B–Rad51C (Fig. 4C, panels II and III). We also examined whether amounts of Rad51B–Rad51C below and above that used before would lead to different levels of homologous DNA pairing and strand exchange. As shown in Figure 5, the optimal concentration of Rad51B–Rad51C was from 0.4 to 1.0 μM, and amounts of Rad51B–Rad51C above the optimal level in fact led to gradual inhibition of the reaction (Fig. 5; data not shown).

One possible explanation for the stimulatory effect of Rad51B–Rad51C complex (Fig. 5) is that this protein complex promotes homologous DNA pairing and strand exchange regardless of the order of addition of RPA. To test this possibility, we used the same amount of Rad51B–Rad51C complex (0.8 μM) that afforded the maximal restoration of DNA pairing and strand exchange (see Fig. 5A,B) in reactions wherein the protein complex was (1) added with Rad51 to the ssDNA substrate, followed by RPA; (2) added with RPA after Rad51 had already nucleated onto the ssDNA substrate; and (3) added after the ssDNA had first been incubated with Rad51 and then with RPA. No measurable effect of the Rad51B–Rad51C complex on the rate of formation of joint molecules and nicked circular duplex was recorded in any of these experiments (data not shown). We also tested whether the inclusion of Rad51B–Rad51C complex would alter the concentration of Rad51 needed for optimal pairing and strand exchange, determined previously to be from 3 to 4 nucleotides per Rad51 monomer (Baumann and West 1997, 1999; Gupta et al. 1997; Sigurdsson et al. 2001). However, Rad51B–Rad51C did not change the concentration of Rad51 needed for optimal reaction efficiency (data not shown).

To examine whether the Rad51B–Rad51C complex can substitute for RPA in the DNA pairing and strand exchange reaction, we used a range of Rad51B–Rad51C concentrations (0.4, 0.8, 1.2, and 2.0 μM) with a fixed concentration of Rad51 (7.5 μM) and ssDNA (30 μM) without RPA. Rad51B–Rad51C did not promote the formation of nicked circular duplex (Fig. 6) even after 60 min of reaction (data not shown), showing that it cannot substitute for RPA, which is highly effective in enabling Rad51 to make nicked circular duplex (Fig. 4; Sigurdsson et al. 2001). Interestingly, we observed an increase in joint molecules at 0.8 and 1.2 μM of Rad51B–Rad51C complex (Fig. 6). Specifically, after 30 min, the level of DNA joint molecules increased from ~5% in the absence of Rad51B–Rad51C to ~10% upon the inclusion of 1.2 μM Rad51B–Rad51C. As with the earlier strand exchange restoration experiment (Fig. 5), suppression of DNA joint molecule formation was seen at higher concentrations of the Rad51B–Rad51C complex (Fig. 6A,B; data not shown). In other experiments, over the range of Rad51B–Rad51C concentration from 3 to 45 nucleotides/protein complex, we did not detect any DNA joint molecule with the ϕX substrates, regardless of whether RPA was present (data not shown). These observations suggested that the Rad51B–Rad51C complex is devoid of homologous DNA pairing activity.

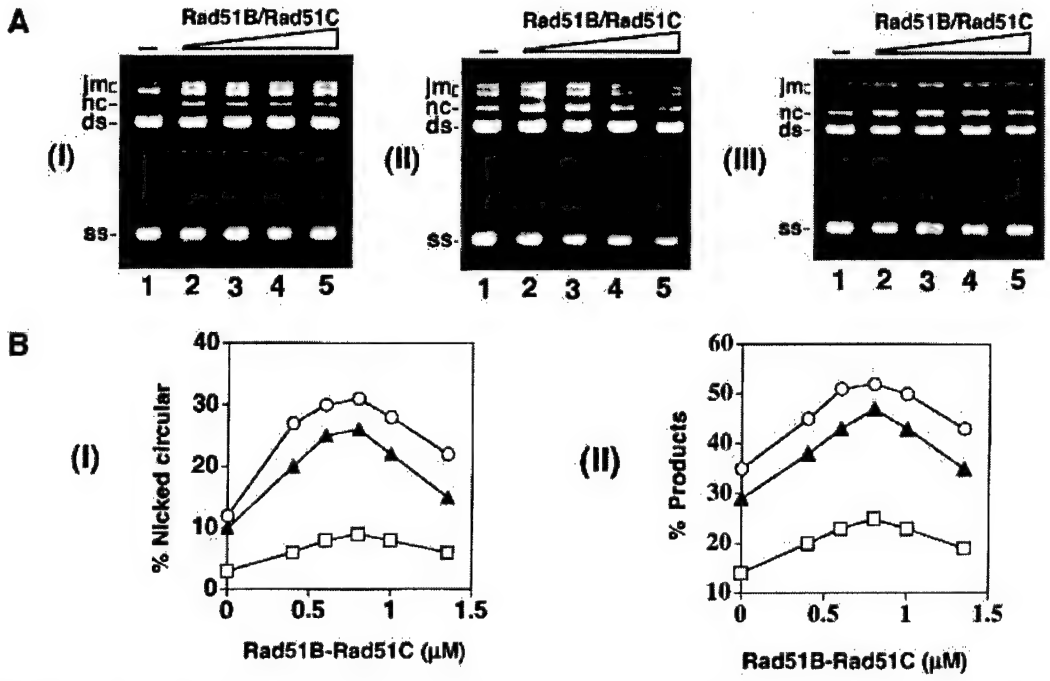

Figure 5. Mediator activity as a function of Rad51B–Rad51C concentration. (*A*) The ϕX174 ssDNA template (30 μM nucleotides) was incubated with Rad51 (7.5 μM), RPA (1.5 μM), and increasing concentrations of Rad51B–Rad51C (0, 0.6, 0.8, 1.0, and 1.4 μM in lanes *1–5*, respectively) for 10 min before the ϕX174 linear duplex (15 μM base pairs) was incorporated to complete the reaction mixtures. Portions of the reaction mixtures were withdrawn at 30 min (panel *I*), 60 min (panel *II*), and 80 min (panel *III*) and then processed for agarose gel electrophoresis. (*B*) The results from *A* and from two other independent experiments performed under the same reaction conditions were compiled and graphed. Symbols: results from the 30 min timepoint (squares), the 60 min timepoint (filled triangles), and the 80 min timepoint (circles). Panel *I* shows the levels of nicked circular duplex formed, and panel *II* shows the amounts of total reaction products (joint molecules and nicked circular duplex).

Discussion

Paradoxical effects of RPA on homologous DNA pairing and strand exchange

Like other members of the RecA/Rad51 class of recombinases (Ogawa et al. 1993; Sung and Robberson 1995; Roca and Cox 1997; Bianco et al. 1998), hRad51 assembles onto ssDNA to form a nucleoprotein filament in an ATP-dependent manner (Benson et al. 1994). Extensive biochemical studies with RecA have indicated that the search for DNA homology, DNA joint formation, and DNA strand exchange all occur within the confines of the RecA-ssDNA nucleoprotein filament. The assembly of the recombinase-ssDNA nucleoprotein filament is therefore the critical first step in the homologous DNA pairing and strand exchange reaction, and is generally referred to as the presynaptic phase of this reaction (Roca and Cox 1997; Bianco et al. 1998; Sung et al. 2000).

For RecA and yeast Rad51, the assembly of the presynaptic filament on plasmid-length DNA molecules is dependent on the cognate single-strand binding protein, *E. coli* SSB or yeast RPA, which acts to minimize the secondary structure in the DNA template and hence renders extension of the nascent nucleoprotein filament facile. In most experimental systems, the single-strand DNA binding factor is added subsequent to the recombinase, that is, after nucleation of the recombinase onto the ssDNA substrate has already commenced. Interestingly, precoating of the ssDNA template with the single-strand binding protein (Umezu et al. 1993; Sugiyama et al. 1997; New et al. 1998; Shinohara and Ogawa 1998) or coincubation of RPA, Rad51, and the ssDNA substrate (Sung 1997a,b) results in pronounced suppression of the reaction efficiency. We have shown in the present study that the hRad51 recombinase activity is similarly suppressed by RPA during the presynaptic phase. Taken together, the results indicate that the single-strand binding protein, while important for secondary structure removal in the ssDNA template, can interfere with the nucleation of the recombinase onto the DNA template and can thus prevent the formation of a contiguous presynaptic filament.

Mediator function in the Rad51B–Rad51C complex

Exploiting the paradoxical behavior of the single-strand DNA binding protein in the assembly of the presynaptic recombinase filament, various recombination mediator proteins capable of overcoming the suppressive effect of the single-strand binding protein have been identified in prokaryotes and yeast cells (Umezu et al. 1993; Sung 1997a,b; New et al. 1998; Shinohara and Ogawa 1998; Beernink and Morrical 1999). In yeast, two such mediators—Rad52 and the Rad55–Rad57 complex—have been

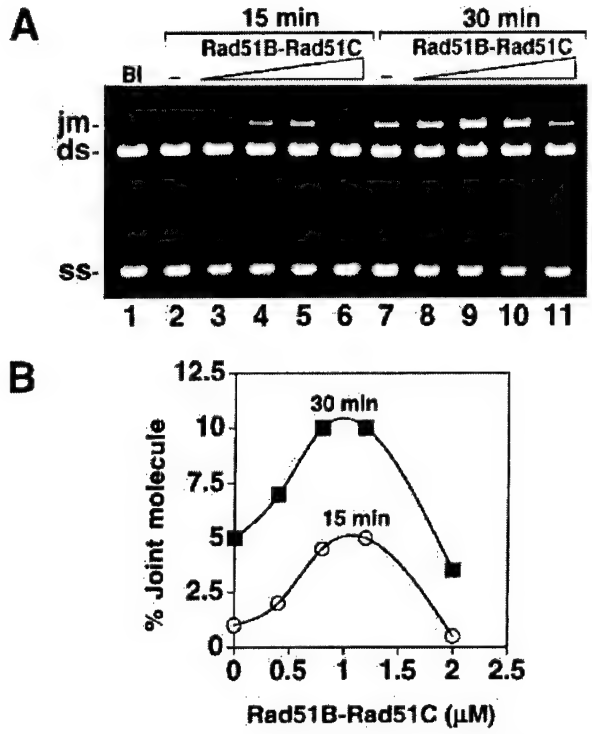

Figure 6. Rad51B–Rad51C stimulates DNA joint formation but does not replace RPA in DNA strand exchange. (*A*) Rad51 and Rad51B–Rad51C were used in the homologous DNA pairing and strand exchange reaction without RPA. The concentrations of the reaction components were: Rad51, 7.5 μM; Rad51B–Rad51C, 0.4 to 2 μM, as indicated; ssDNA, 30 μM nucleotides; linear duplex, 15 μM base pairs. (*B*) Graphic representation of the results from the experiment in *A*.

described. Rad55 and Rad57 are similar to Rad51B and Rad51C in that they too exhibit homology to Rad51 and form a stable stoichiometric complex via direct interaction (Sung et al. 2000). Importantly, both Rad51B–Rad51C and Rad55–Rad57 complexes are needed for the assembly of Rad51 nuclear foci in response to DNA damaging treatment (Gasior et al. 1998, 2001; Takata et al. 2000, 2001), which are thought to correspond to the sites of ongoing DNA damage repair.

We show here that purified Rad51B–Rad51C complex has single-strand DNA binding and ssDNA-stimulated ATPase activities. Importantly, Rad51B–Rad51C can promote the Rad51-mediated homologous DNA pairing and strand exchange reaction under conditions wherein Rad51 must contend with the competition by RPA for binding sites on the initiating ssDNA substrate. In addition, DNA joint formation by Rad51 in the absence of RPA is also enhanced by Rad51B–Rad51C. We asked whether Rad51B–Rad51C could stimulate the rate of DNA pairing and strand exchange, but did not uncover a postsynaptic role (Roca and Cox 1997; Bianco et al. 1998; Sung et al. 2000) in this protein complex. Rad51B–Rad51C complex did not pair the ϕX DNA substrates used, nor did it lower the concentration of Rad51 needed to attain optimal reaction efficiency. Thus, the results of our study suggest a specific role of Rad51B–Rad51C in promoting the assembly of the presynaptic Rad51 filament, identifying it as a mediator of recombination, in a manner analogous to what has been described for the yeast Rad55–Rad57 complex (Sung et al. 2000). Given that both Rad51B and Rad51C are indispensable for recombination and DNA repair and needed for the normal assembly of Rad51 nuclear foci after DNA damaging treatment, we surmise that the Rad51B–Rad51C mediator function likely contributes to the recruitment of hRad51 to sites of recombination and DNA damage repair.

Role of other Rad51 paralogs in recombination processes

Aside from Rad51B and Rad51C, three additional Rad51 paralogs, namely, XRCC2, XRCC3, and Rad51D, have been described (Thompson and Schild 2001). XRCC3 and Rad51C also form a stable complex that has DNA binding activity (Kurumizaka et al. 2001; Masson et al. 2001). Rad51D (also called Rad51L3) has DNA binding and ssDNA-stimulated ATPase activities, and it forms a stable complex with XRCC2 (Braybrooke et al. 2000). Whether the XRCC2–Rad51D and XRCC3–Rad51C complexes also have a recombination mediator function remains to be determined. However, that the formation of DNA damage-induced Rad51 nuclear foci is impaired in mutants of all five of the Rad51 paralogs would seem to suggest that they all play a role in the delivery of Rad51 to DNA lesions in vivo. It thus seems possible that some combination of complexes of the Rad51 paralogs could have an enhanced mediator function in the Rad51-catalyzed homologous DNA pairing and strand exchange reaction. In fact, our observation that Rad51B–Rad51C only partially overcomes the suppressive effect of RPA is consistent with such a scenario. It remains to be seen whether the other two Rad51 paralog pairs, either by themselves or in combination with each other and with Rad51B–Rad51C, may also function in the postsynaptic phase of the pairing and strand exchange reaction by enhancing the efficiency of DNA joint formation and promoting DNA branch migration.

Materials and methods

Antibodies

Rad51B protein was expressed in *E. coli* BL21 (DE3) using the T7 promoter in the vector pET11a. The insoluble Rad51B protein was purified by preparative SDS-PAGE, dialyzed into phosphate-buffered saline (10 mM NaH_2PO_4 at pH 7.2, 150 mM NaCl), and then used as antigen for production of antisera in rabbits. A GST–Rad51C fusion protein was expressed in *E. coli* BL21 (DE3) using the vector pGEX-2T. The GST–Rad51C fusion protein is also insoluble and was purified for antibody production in rabbits as described for Rad51B. The antigens were covalently conjugated to cyanogen bromide activated Sepharose 4B (Pharmacia-LKB) for use as affinity matrices to purify monospecific antibodies from rabbit antisera (Sung et al. 1987).

DNA substrates

ϕX174 viral (+) strand was purchased from New England Biolabs, and the ϕX174 replicative form I DNA was from GIBCO BRL. The replicative form I DNA was linearized with *Apa*LI. All of the DNA substrates were stored in TE (10 mM Tris-HCl at pH 7.5, 0.5 mM EDTA).

Fractionation of HeLa cell extract

To make extract, 10.4 g of HeLa S3 cells (National Cell Culture Center) was suspended in 15 mL of cell breakage buffer (50 mM Tris/HCl at pH 7.5, 2 mM EDTA, 10% sucrose, 100 mM KCl, 1 mM dithiothreitol and the following protease inhibitors: aprotinin, chymostatin, leupeptin, pepstatin, all at 3 μg/mL, and 1 mM phenylmethylsulfonyl fluoride) and passed through a French Press at 20,000 psi. After centrifugation (100,000*g* for 1 h), the clarified extract (20 mL) was loaded onto a column of Q-Sepharose (4 mL), which was fractionated with a 45 mL gradient of KCl from 100 to 600 mM in K buffer (20 mM KH_2PO_4 at pH 7.4, 0.5 mM EDTA, 1 mM DTT, and 10% glycerol) collecting 30 fractions. To identify the Rad51B and Rad51C proteins, the Q-Sepharose fractions (8 μL) were subjected to immunoblot analyses with anti-Rad51B and anti-Rad51C antibodies after SDS-PAGE in 11% gels.

Expression of Rad51B and Rad51C in yeast cells

The *RAD51B* gene was placed under the control of the *GAL-PGK* promoter in vector pPM231 (*2μ, GAL-PGK, LEU2d*) to yield pRad51B.1 (*2μ, GAL-PGK-RAD51B, LEU2d*) and *RAD51C* were placed under the control of the *PGK* promoter in vector pPM255 (*2μ, PGK, URA3*) to yield pRad51C.1 (*2μ, PGK-RAD51C, URA3*). The empty expression vector, pR51B.1, and pR51C.1 were introduced into the protease-deficient yeast strain BJ5464 (*MATα, ura3-52, trp-1, leu2Δ1, his3Δ200, pep4::HIS3, prbΔ1.6R*). Induction of Rad51B and Rad51C followed the protocol of Petukhova et al. (2000). Extracts (2 mL of buffer per gram of yeast cells) were prepared using a French press and clarified by centrifugation, as described above.

Purification of the Rad51B–Rad51C complex

Six-histidine (6His)-tagged Rad51B and untagged Rad51C recombinant baculoviruses were constructed by cloning the cDNAs for these proteins into the baculovirus transfer vector pVL1393 (Invitrogen). Amplification of the recombinant viruses was carried out in Sf9 cells and for protein expression, High-Five insect cells (Invitrogen) were infected with the 6His-tagged Rad51B and Rad51C baculoviruses at an MOI of 5. The insect cells were harvested 36–48 h postinfection. For the purification of the Rad51B–Rad51C complex, High-Five cells were coinfected with the Rad51B and Rad51C baculoviruses at an MOI of 5 for both viruses. The insect cells were harvested 36–48 h postinfection.

All the protein purification steps were carried out at 0 to 4°C. Cell lysate was prepared from 1 L of insect cell culture (15 g) using a French press in 60 mL of cell breakage buffer. The lysate was clarified by centrifugation (100,000*g* for 1 h) and the supernatant was applied on a 10 mL SP-Sepharose column. The flow-through from SP-Sepharose was then fractionated in a column of Q-Sepharose (10 mL of matrix) with a 30 mL, 100 to 600 mM KCl gradient in buffer K. Rad51B–Rad51C eluted from 200–300 mM KCl, and the peak fractions were pooled and mixed with 0.6 mL of Ni-NTA agarose for 2 h, followed by fractionation with a 15 mL, 0 to 250 mM imidazole in buffer K. The Rad51B–Rad51C peak fractions were pooled and further fractionated in a column of Macro-hydroxyapatite (0.5 mL matrix) with a 15 mL, 0 to 180 mM KH_2PO_4 gradient in buffer K. The peak fractions were pooled and dialyzed against buffer K with 50 mM KCl and applied onto a Mono Q column (HR5/5), which was developed with a 15 mL gradient from 100 to 800 mM KCl in buffer K. Rad51B–Rad51C from the Mono Q step was concentrated in a Centricon-30 microconcentrator to 2 mg/mL and stored at −70°C.

Immunoprecipitation

Affinity-purified anti-Rad51B, anti-Rad51C, and anti-ySrs2 antibodies, 1.0 mg each, were coupled to 300 μL protein A agarose beads as described previously (Sung 1997a). In Figure 1C, 0.3 mL of the Q-Sepharose pool (fractions 8 to 16) containing the Rad51B–Rad51C peak was mixed with 10 μL of protein A beads containing anti-Rad51B, anti-Rad51C, or anti-ySrs2 antibodies at 4°C for 5 h. The beads were washed twice with 200 μL of buffer K containing 1 M KCl and once with 200 μL of buffer K, before being eluted with 20 μL of 2% SDS at 37°C for 10 min. The SDS eluates, 2 μL each, were analyzed by immunoblotting to reveal their content of Rad51B and Rad51C.

Affinity binding of the Rad51B–Rad51C complex to nickel-NTA agarose

Extract from 0.5 mL packed cell volume of High-Five insect cells harboring either the six-histidine-tagged Rad51B or Rad51C recombinant baculovirus was prepared as described above, using 3 mL of cell breakage buffer. After ultracentrifugation (100,000*g* for 1 h), 0.25 mL of the Rad51B and Rad51C containing extracts were mixed either with each other or with 0.25 mL of extract from insect cells that did not contain any recombinant baculovirus. After incubation on ice for 1 h, the various mixtures were subjected to ultracentrifugation (100,000*g* for 1h), and then rocked gently with 50 μL of nickel-NTA agarose beads (QIAGEN) at 4°C for 3 h. The beads were washed sequentially with 0.2 mL of 10 mM and 20 mM imidazole, and then with 0.1 mL of 150 mM imidazole. The starting fractions, supernatants after nickel-binding, and the three imidazole washes, 5 μL each, were subjected to immunoblot analysis to determine their content of Rad51B and Rad51C.

Other recombination proteins

Human Rad51 (hRad51 Lys^{313}) was expressed in and purified from *E. coli* as described (Sigurdsson et al. 2001). Human RPA was purified from *E. coli* cells transformed with a plasmid that co-overexpresses the three subunits of this factor (Henricksen et al. 1994), as described (Sigurdsson et al. 2001). Both hRad51 and hRPA were nearly homogeneous.

Homologous DNA pairing and strand exchange reactions

All the reaction steps were carried out at 37°C. The reaction buffer was 40 mM Tris-HCl (pH 7.8), 2 mM ATP, 1 mM $MgCl_2$, and 1 mM DTT, and contained an ATP regenerating system consisting of 8 mM creatine phosphate and 28 μg/mL creatine kinase. The standard reaction had a final volume of 12.5 μL, and was assembled by first incubating hRad51 (7.5 μM) added in 0.5 μL of storage buffer and ϕX174 viral (+) strand (30 μM nucleotides) added in 1 μL for 5 min. After this, hRPA (1.5 μM) in 0.5 μL of storage buffer was added and following a 5 min incubation, 1.25 μL of ammonium sulfate (1 M stock, final concentration of

100 mM) was incorporated. Immediately afterward, linear ϕX174 replicative form I DNA (15 μM base pairs) in 1 μL was added to complete the reaction. At the indicated times, 5 μL portions were withdrawn, mixed with 7.5 μL of 0.8% SDS and 800 μg/mL proteinase K, incubated for 15 min at 37°C, before being run in 0.9% agarose gels in TAE buffer (40 mM Tris acetate at pH 7.4, 0.5 mM EDTA). The gels were stained in ethidium bromide (2 μg/mL in H_2O) for 1 h, destained for 12 to 18 h in a large volume of water at 4°C, and then subjected to image analysis in a NucleoTech gel documentation station equipped with a CCD camera. In some experiments, the reaction was scaled up appropriately to accommodate the increased number of timepoints used. In Figure 4B, panel II, storage buffer was added instead of RPA, but otherwise the reaction was assembled in exactly the same manner as the standard reaction. In Figure 5A, panel I, Rad51 and RPA were added together to ssDNA at the beginning of the reaction and incubated for 10 min with the latter, but otherwise the additions of the ammonium sulfate and linear ϕX duplex followed the procedure described for the standard reaction. In Figure 5A, panel II and in Figure 5B, Rad51, RPA, and the indicated amounts of Rad51B–Rad51C were incubated with the ssDNA for 10 min, but otherwise the additions of the ammonium sulfate and linear ϕX duplex followed the procedure described for the standard reaction. In Figure 6, Rad51 and the indicated amounts of Rad51B–Rad51C were incubated with the ssDNA for 10 min, but otherwise the additions of the ammonium sulfate and linear ϕX duplex followed the procedure described for the standard reaction.

DNA mobility shift and ATPase assays

The indicated amounts of Rad51B–Rad51C complex were incubated with ϕX ssDNA (12 μM nucleotides), dsDNA (4 μM base pairs), or both ssDNA (12 μM nucleotides) and dsDNA (4 μM base pairs) in reaction buffer (50 mM Tris-HCl at pH 7.8, 1 mM DTT, 100 μg/mL BSA, 1 mM $MgCl_2$, 1 mM ATP, and 100 mM KCl) for 10 min at 37°C. After electrophoresis in 0.9% agarose gels in TAE buffer at 4°C, the gels were stained in ethidium bromide (2 μg/mL in H_2O) for 1 h and destained at 4°C for 12 to 18 h, before being subjected to image analysis in the gel documentation station.

Rad51B–Rad51C (1.8 μM) was incubated in the absence or presence of ssDNA (20 μM nucleotides) or dsDNA (20 μM base pairs) in 10 μL of reaction buffer (50 mM Tris-HCl at pH 7.8, 1 mM DTT, 1 mM $MgCl_2$) containing 1 mM [γ-^{32}P]ATP for the indicated times at 37°C. The level of ATP hydrolysis was determined by thin layer chromatography as described previously (Petukhova et al. 2000).

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Homologous DNA Pairing by Human Recombination Factors Rad51 and Rad54*

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Human Rad51 (hRad51) and Rad54 proteins are key members of the *RAD52* group required for homologous recombination. We show an ability of hRad54 to promote transient separation of the strands in duplex DNA via its ATP hydrolysis-driven DNA supercoiling function. The ATPase, DNA supercoiling, and DNA strand opening activities of hRad54 are greatly stimulated through an interaction with hRad51. Importantly, we demonstrate that hRad51 and hRad54 functionally cooperate in the homologous DNA pairing reaction that forms recombination DNA intermediates. Our results should provide a biochemical model for dissecting the role of hRad51 and hRad54 in recombination reactions in human cells.

In eukaryotic organisms, the repair of DNA double-stranded breaks by homologous recombination is mediated by a group of evolutionarily conserved genes known as the *RAD52* epistasis group. Members of the *RAD52* group (*RAD51*, *RAD52*, *RAD54*, *RAD55*, *RAD57*, *RAD59*, and *RDH54/TID1*) were first uncovered in genetic screens in the budding yeast *Saccharomyces cerevisiae* (1, 2). In mammals, the efficiency of homology-directed recombinational DNA repair is modulated by the tumor suppressors BRCA1 and BRCA2 (3), providing a compelling link between this DNA repair pathway and the suppression of tumor formation. The involvement of the homologous recombination machinery in the maintenance of genome stability and tumor suppression underscores the need for deciphering the action mechanism of this machinery.

During the recombinational repair of DNA double-stranded breaks, a single-stranded DNA intermediate is utilized by the recombination machinery to invade a DNA homolog, most often the sister chromatid, to form a DNA joint molecule referred to as a D-loop (2). D-Loop formation is critical for subsequent steps in the recombination reaction, which include repair DNA synthesis and resolution of recombination intermediates (1, 2), that lead to the restoration of the integrity of the injured chromosome.

In the past several years, biochemical studies have begun to shed light on the functions of the human *RAD52* group proteins in DNA joint formation. Much of the published work has centered on the human Rad51 (hRad51)[1] protein, which is structurally related to the *Escherichia coli* recombinase enzyme RecA (4). Like RecA, hRad51 assembles into a right-handed filament on single-stranded (ss) DNA in a reaction that is dependent on ATP binding (reviewed in Ref. 5). Importantly, hRad51 protein has been shown to have DNA pairing and strand exchange activities that yield DNA joints between homologous ssDNA and double-stranded DNA substrates (6–8). The homologous pairing and strand exchange function of hRad51 is augmented by replication protein A (RPA), a heterotrimeric single-stranded DNA binding factor (6, 8), by hRad52 protein (9), and by the Rad51B-Rad51C heterodimeric complex (10), which is the functional equivalent of the yeast Rad55-Rad57 complex (11).

The *RAD54* encoded product belongs to the Swi2/Snf2 protein family (12). Purified hRad54 exhibits DNA-dependent ATPase and DNA supercoiling activities (13–15). However, the manner in which hRad54 influences the hRad51-mediated recombination reaction has remained mysterious. Here we report our biochemical studies that show functional interactions between hRad51 and hRad54 in DNA supercoiling and homologous DNA pairing reactions. We discuss how hRad51 and hRad54 cooperate to make DNA joints during recombination processes.

EXPERIMENTAL PROCEDURES

Anti-Rad54 Antibodies—The first 238 amino acid residues of the human Rad54 protein were fused to glutathione *S*-transferase in the vector pGEX-3X. The fusion protein was expressed in *E. coli* strain BL21 (DE3) and purified from inclusion bodies by preparative denaturing polyacrylamide gel electrophoresis and used as antigen for raising polyclonal antibodies in rabbits. The same antigen was covalently conjugated to cyanogen bromide-activated Sepharose 4B and used as affinity matrix to purify the antibodies from rabbit antisera, as described (16).

Rad54 Expression and Purification—A recombinant baculovirus containing the cloned *hRad54* cDNA with an added FLAG epitope at the C terminus was generated. HighFive insect cells were infected with the recombinant baculovirus at a multiplicity of infection of 10 and harvested after 48 h of incubation. An extract was prepared from 500 ml of insect cell culture (5×10^8 cells) using a French Press in 60 ml of cell breakage buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 10% sucrose, 200 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and the following protease inhibitors at 3 μg/ml each: aprotinin, chymostatin, leupeptin, and pepstatin). After centrifugation (100,000 × *g* for 60 min), the clarified extract was loaded onto a Q-Sepharose column (10-ml matrix). The flow-through fraction from the Q column was fractionated in a sulfopropyl-Sepharose column (10-ml matrix) with a 50-ml, 0–700

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[1] The abbreviations used are: hRad51, human Rad51; yRad51, yeast Rad51; ss, single-stranded; BSA, bovine serum albumin; AMP-PNP, adenosine 5′-(β,γ-imino)triphosphate; ATPγS, adenosine 5′-3-*O*-(thio)-triphosphate.

mM KCl gradient in K buffer (20 mM KH_2PO_4 at pH 7.4, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol). Fractions containing the peak of hRad54 were pooled and loaded onto a 1-ml Macro-hydroxyapatite (Bio-Rad) column, which was eluted with 30 ml of 0–300 mM KH_2PO_4 in K buffer. The peak fractions were pooled and mixed with 1.5 ml of Anti-FLAG M2 agarose (Sigma) and rocked for 3 h at 4 °C. The FLAG agarose was washed three times with 3 ml of 150 mM KCl in buffer K before eluting hRad54 using the same buffer containing 1 mg/ml of the FLAG peptide (Sigma). hRad54 (~1 mg) eluted from the FLAG matrix was concentrated in a Centricon-30 microconcentrator to 5 mg/ml and stored in small aliquots at −70 °C.

Rad51 and hrad51 K133R Proteins—The hRad51 protein was expressed in the *E. coli* RecA-deficient strain BLR (DE3) and purified to near homogeneity using our previously described procedure (8). The hrad51 K133R mutant was expressed and purified to near homogeneity in exactly the same way.

Topoisomerase I—*E. coli* topoisomerase I was purified to near homogeneity from the *E. coli* strain JM101 with plasmid pJW312-sal containing the *topA* gene under the Lac promoter, as described (17).

Binding of hRad54 to Affi-hRad51 Beads—Purified hRad51 and bovine serum albumin (BSA) were coupled to Affi-Gel 15 beads at 4 °C following the instructions of the manufacturer (Bio-Rad). The resulting matrices contained 4 and 12 mg/ml hRad51 and BSA, respectively. Purified hRad54 (1.2 μg) was mixed with 5 μl of Affi-Rad51 or Affi-BSA at 4 °C for 30 min in 30 μl of buffer containing 100 mM KCl and 0.1% Triton X-100 by constant tapping. The beads were washed twice with 50 μl of the same buffer before being treated with 30 μl of 2% SDS at 37 °C for 5 min to elute the bound hRad54. The various fractions (4 μl each) were analyzed by immunoblotting to determine their content of hRad54.

DNA Substrates—Topologically relaxed φX174 DNA was prepared as described (18), and pBluescript SK DNA was made in *E. coli* DH5α and purified as described (19). The oligonucleotide used in the D-loop reaction is complementary to positions 1932–2022 of the pBluescript SK DNA and had the sequence 5′-AAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTT-3′. This oligonucleotide was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

ATPase Assay—The hRad54 protein (60 nM) was incubated with replicative form I φX174 DNA (30 μM base pairs) and 1.5 mM [γ-^{32}P]ATP with or without 400 nM hRad51 or yRad51 in 10 μl of reaction buffer (20 mM Tris-HCl, pH 7.4, 25 mM KCl, 1 mM dithiothreitol, 4 mM $MgCl_2$, 100 μg/ml BSA) at 30 °C for the indicated times. The level of ATP hydrolysis was determined by thin layer chromatography, as described (19).

DNA Supercoiling and DNA Strand-opening Reactions—Increasing amounts of hRad54 were incubated with 80 ng of relaxed φX174 DNA (12 μM nucleotides) for 2 min at 23 °C in 12 μl of reaction buffer (20 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM dithiothreitol, 100 μM ATP, and an ATP-regenerating system consisting of 10 mM creatine phosphatase and 28 μg/ml creatine kinase). Following the addition of 100 ng of *E. coli* topoisomerase I in 0.5 μl, the reactions were incubated for 10 min at 23 °C and then deproteinized by treatment with 0.5% SDS and proteinase K (0.5 mg/ml) for 10 min at 37 °C. Samples were run on 1% agarose gels in TAE buffer (35 mM Tris acetate, pH 7.4, 0.5 mM EDTA) at 23 °C and then stained with ethidium bromide. In the experiment in Fig. 4*B*, the relaxed DNA was incubated with the indicated amounts of hRad51 and hRad54 for 2 min at 23 °C, followed by the addition of topoisomerase and a 10-min incubation at 23 °C. For the P1 sensitivity experiments in Figs. 3*C* and 4*C*, the reactions were assembled in the same manner except that 0.4 unit of P1 nuclease (Roche) was used instead of topoisomerase. The DNA species were resolved in a 1% agarose gel containing 10 μM ethidium bromide in TAE buffer.

D-Loop Reaction—For the time course reactions (25 μl, final volume) in Fig. 5, hRad51 or hrad51 K133R (800 nM) was incubated with the 5′-labeled ss oligonucleotide (2.5 μM nucleotides) for 3 min at 37 °C in 22 μl of reaction buffer (20 mM Tris-HCl, pH 7.4, 100 μg/ml BSA, 1.5 mM $MgCl_2$, 2 mM ATP, and the ATP-generating system described above). This was followed by the addition of hRad54 (120 nM) in 1 μl and incubation at 23 °C for 2 min. The reaction was completed by adding the pBluescript SK replicative form DNA (35 μM base pairs) in 2 μl. The reaction mixture was incubated at 30 °C, and 3.8-μl aliquots were withdrawn at the indicated times, deproteinized, and run in 1% agarose gels in TAE buffer. The gels were dried and the levels of D-loop were quantified by phosphorimage analysis. The reactions in which ATP, hRad51, or hRad54 was omitted or ATP was replaced by ATPγS or AMP-PNP were scaled down 2-fold to a 12.5-μl final volume, but they were otherwise assembled and processed in exactly the same manner.

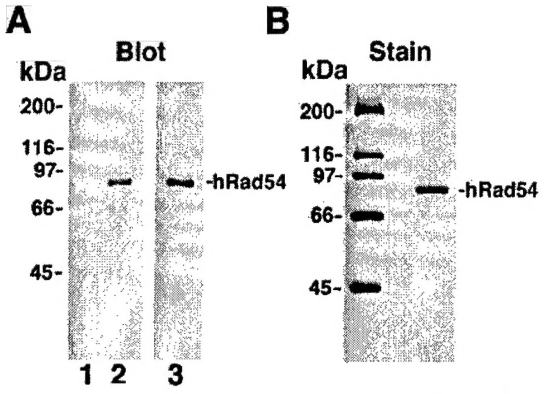

FIG. 1. **Purification of hRad54.** *A*, expression of hRad54 in insect cells. Extracts from uninfected insect cells (*lane 1*) and from insect cells infected with the recombinant hRad54 baculovirus (*lane 2*) were probed with affinity-purified anti-hRad54 antibodies. In *lane 3*, 100 ng of purified hRad54 was also subjected to immunoblotting. *B*, purified hRad54 protein (1 μg) was run in an 8% denaturing polyacrylamide gel and stained with Coomassie Blue.

RESULTS

Human Recombination Factors—Human Rad51 was expressed in a recA-*E. coli* strain and purified to near homogeneity as described previously (8). The hrad51 K133R mutant, which harbors the change of the conserved lysine residue in the Walker type A nucleotide binding motif to arginine, was also similarly expressed and purified. In agreement with previously published results (20), hrad51 K133R has negligible ATPase activity compared with wild type hRad51 (data not shown). We cloned the human *RAD54* cDNA from a testis cDNA library using the polymerase chain reaction. The entire cloned *hRAD54* cDNA was sequenced to ensure that it agreed with the published sequence (21). We tagged hRad54 protein with a FLAG epitope at the carboxyl terminus and expressed it in insect cells by the use of a recombinant baculovirus (Fig. 1*A*). We obtained ~1 mg of nearly homogeneous hRad54 (Fig. 1*B*) from 500 ml of insect cell culture by a combination of conventional column chromatography and affinity binding to an antibody specific for the FLAG epitope. The purified hRad54 has a level of DNA-dependent ATPase very similar to that described in the literature (13) (see later).

hRad54 Physically Interacts with hRad51—Golub *et al.* (22) found that the amino terminus of hRad54 can bind hRad51 in *in vitro* analyses and also in the yeast two-hybrid system. However, in mouse embryonic stem cells, association of mRad51 and mRad54 requires prior treatment of cells with a DNA damaging agent (14). To examine whether purified hRad54 physically interacts with hRad51, we coupled hRad51 to Affi-Gel beads to use as affinity matrix for binding hRad54. As shown in Fig. 2, purified hRad54 was efficiently retained on Affi-hRad51 beads but not on Affi-beads that contained BSA. When a less purified hRad54 fraction (~25% hRad54) was used, hRad54, but not the contaminating protein species, bound to the Affi-hRad51 beads (data not shown). The results thus indicate a direct and specific interaction between hRad51 and hRad54.

DNA Supercoiling and DNA Strand Opening by hRad54—Tan *et al.* (14) showed an ability of hRad54 to alter the DNA linking number of a nicked plasmid in the presence of DNA ligase. The induction of DNA linking number change was dependent on ATP hydrolysis by hRad54 (14). The same group also used scanning force microscopy to provide evidence that hRad54 tracks on DNA when ATP is hydrolyzed (15). A schematic depicting the basis for tracking-induced DNA supercoiling by hRad54 is given in Fig. 3*A*.

The yRad54 protein also tracks on DNA and, as a result,

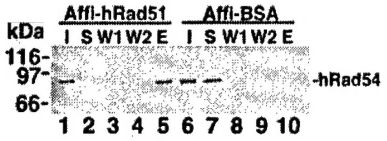

FIG. 2. **hRad54 interacts with hRad51.** Purified hRad54 (1.2 μg) was mixed with Affi-beads containing either BSA (Affi-BSA) or hRad51 (Affi-Rad51) in 30 μl and washed twice with 50 μl buffer, followed by treatment of the beads with 30 μl of SDS to elute bound hRad54. The starting material (*I*), supernatant (*S*), the two washes (*W1* and *W2*), and the SDS eluate (*E*), 4 μl each, were subjected to immunoblotting to determine their hRad54 content.

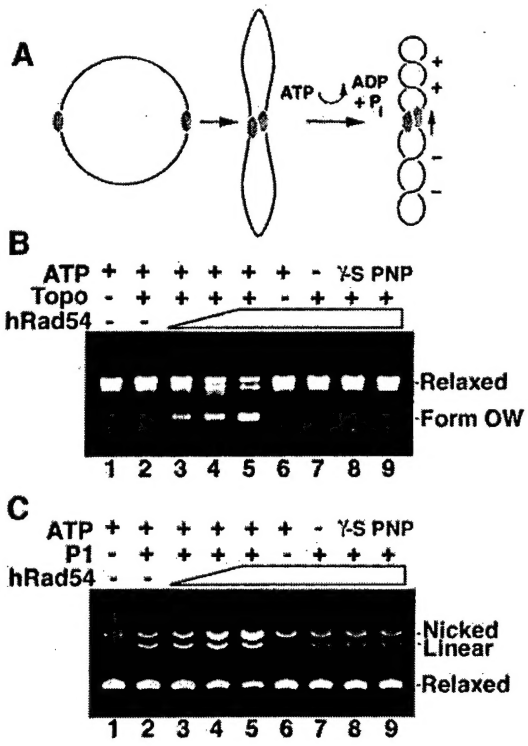

FIG. 3. **hRad54 supercoils DNA and promotes DNA strand opening.** *A*, basis for hRad54-induced supercoiling, as per Ristic *et al.* (15) and Van Komen *et al.* (18). The free energy from ATP hydrolysis fuels the tracking of a hRad54 oligomer on DNA, producing a positively supercoiled domain ahead of protein movement and a negatively supercoiled domain behind. *B*, increasing amounts of hRad54 (200, 400, and 750 nM in *lanes 3–5*, respectively) were incubated with topologically relaxed DNA (20 μM nucleotides) and *E. coli* topoisomerase I in buffer that contained ATP. The highest amount of hRad54 (750 nM) was also incubated with the DNA substrate in the absence of topoisomerase (*lane 6*) and in the presence of topoisomerase but with the omission of ATP (*lane 7*) or the substitution of ATP by ATPγS (γ-*S*; *lane 8*) and AMP-PNP (*PNP*; *lane 9*). DNA alone (*lane 1*) or DNA incubated with topoisomerase (*lane 2*) was also included. The reaction mixtures were run in a 1% agarose gel, which was treated with ethidium bromide to reveal the DNA species. *C*, increasing amounts of hRad54 (200, 400, and 750 nM in *lanes 3–5*, respectively) were incubated with topologically relaxed DNA (20 μM nucleotides) and P1 nuclease in buffer that contained ATP. The highest amount of hRad54 (750 nM) was also incubated with the DNA substrate in the absence of P1 (*lane 6*) and in the presence of P1 but with the omission of ATP (*lane 7*) or the substitution of ATP by ATPγS (γ-*S*; *lane 8*) and AMP-PNP (*PNP*; *lane 9*). DNA alone (*lane 1*) and DNA incubated with P1 in the absence of hRad54 (*lane 2*) were also included. The reaction mixtures were run in a 1% agarose gel containing 10 μM ethidium bromide.

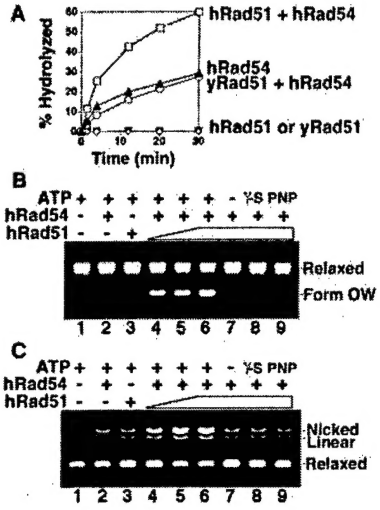

FIG. 4. **The hRad54 activities are stimulated by hRad51.** *A*, hRad54 was incubated with ϕX replicative form I DNA (30 μM nucleotides) and 1.5 mM [γ-^{32}P]ATP for the indicated times, and the level of ATP hydrolysis was quantified by thin layer chromatography. ATPase activity was also measured for hRad51 alone, yRad51 alone, and the combinations of hRad54/hRad51 and hRad54/yRad51. The protein concentrations were as follows: hRad54, 60 nM; hRad51, 400 nM; yRad51, 400 nM. In every case, negligible ATP hydrolysis was seen when DNA was omitted (data not shown). *B*, relaxed DNA (20 μM nucleotides) was incubated with hRad54 (75 nM in *lanes 2* and *4–9*) and hRad51 (80, 160, and 240 nM in *lanes 4–6*, respectively) in the presence of ATP and *E. coli* topoisomerase I. The highest amount of hRad51 (240 nM) was incubated with substrate and topoisomerase I but without hRad54 (*lane 3*) and also with hRad54 (75 nM) but with the omission of ATP (*lane 7*) or its substitution with ATPγS (γ-*S*; *lane 8*) or AMP-PNP (*PNP*; *lane 9*). DNA alone was analyzed in *lane 1*. After deproteinization, the reaction mixtures were run in a 1% agarose gel, which was treated with ethidium bromide to stain the DNA species. *C*, relaxed DNA was incubated with hRad54 (75 nM in *lanes 2* and *4–9*) and hRad51 (80, 160, and 240 nM in *lanes 4–6*, respectively) in the presence of ATP and P1 nuclease. The highest amount of hRad51 (240 nM) was incubated with substrate and P1 but without hRad54 (*lane 3*) and also with hRad54 (75 nM) but with the omission of ATP (*lane 7*) or its substitution with ATPγS (γ-*S*; *lane 8*) or AMP-PNP (*PNP*; *lane 9*). DNA alone was run in *lane 1*. Analysis was carried out in a 1% agarose gel that contained 10 μM ethidium bromide.

generates positive and negative supercoils in the DNA substrate. Removal of the negative supercoils by treatment with *E. coli* topoisomerase I leads to the accumulation of positive supercoils and the formation of an overwound DNA species called Form OW (18). Here we used the same strategy to examine the ability of hRad54 to supercoil DNA. As shown in Fig. 3*B*, in the presence of topoisomerase, purified hRad54 protein readily induces a linking number change in the DNA (18). The DNA supercoiling reaction is dependent on ATP hydrolysis, as revealed by its omission or substitution with a nonhydrolyzable analogue (ATPγS or AMP-PNP) (Fig. 3*B*).

We asked whether the negative supercoils generated as a result of hRad54 tracking on the DNA substrate (15) (Fig. 3, *A* and *B*) leads to transient DNA strand opening by examining the sensitivity of a relaxed DNA template to the single-strand specific nuclease P1, as per Van Komen *et al.* (18). Fig. 3*C* shows that incubation of topologically relaxed DNA with hRad54 rendered the relaxed DNA substrate sensitive to P1 nuclease, as indicated by the accumulation of nicked circular and linear DNA forms. The DNA strand opening reaction is also completely dependent on ATP hydrolysis (Fig. 3*C*).

Activities of hRad54 Are Stimulated by hRad51—The results presented here (Fig. 2) and elsewhere (22) have unveiled a specific interaction between hRad51 and hRad54. We examined whether the hRad54 ATPase would be enhanced upon interaction with hRad51. As shown in Fig. 4*A*, a much higher rate of ATP hydrolysis was seen when hRad54 was combined with hRad51. The fact that yRad51 was ineffective in this reaction (Fig. 4*A*) indicates that the action of hRad51 is specific. Although hRad51 is known to have a weak ATPase activity (23), the fact that the hrad51 K133R mutant protein, which binds but does not hydrolyze ATP (20), was just as effective in

promoting ATP hydrolysis (data not shown) strongly indicated that the increase in ATP hydrolysis was because of enhancement of the hRad54 ATPase function.

We next asked whether the DNA supercoiling activity of hRad54 would also be up-regulated by hRad51. The results showed that hRad51 stimulates the supercoiling reaction, as indicated by a much higher level of Form OW DNA (Fig. 4*B*). Because negative supercoiling generated by hRad54 leads to DNA strand opening (Fig. 3*C*), we thought that hRad51 might also promote this activity. Indeed, the inclusion of hRad51 greatly elevated the nicking of the relaxed DNA substrate by P1 nuclease (Fig. 4*C*). Even with the inclusion of hRad51, no Form OW DNA or nicking of DNA was seen when ATP was omitted or substituted by the nonhydrolyzable analogues ATPγS and AMP-PNP (Fig. 4, *B* and *C*). Thus, the results revealed that hRad51 markedly stimulates the ability of Rad54 to supercoil DNA and unwind DNA strands. The hrad51 K133R protein was just as effective as wild type hRad51 in enhancing the DNA supercoiling and strand opening activities of hRad54 (data not shown). Furthermore, we found that yRad51 does not stimulate the hRad54 activities (data not shown), thus indicating a high degree of specificity in the hRad51 action.

hRad51 and hRad54 Cooperate in Homologous DNA Pairing—The RecA/Rad51 class of general recombinases is central to recombination processes by virtue of their ability to catalyze the homologous DNA pairing reaction that yields heteroduplex DNA joints (2, 24). Because hRad51 and hRad54 physically interact (22) (Fig. 2) and hRad51 enhances the various activities of hRad54 (Fig. 4), it was of considerable interest to examine the influence of hRad54 on hRad51-mediated homologous DNA pairing.

The homologous pairing assay monitors the incorporation of a ^{32}P-labeled single-stranded oligonucleotide into a homologous supercoiled target (pBluescript) to give a D-loop structure (Fig. 5*A*). As reported before (25) and reiterated here (Fig. 5*B*), hRad51 by itself is not particularly adept at forming D-loop. Importantly, the inclusion of hRad54 rendered D-loop formation possible. D-Loop formation by the combination of hRad51 and hRad54 requires ATP hydrolysis, because no D-loop was seen when ATP was omitted or when it was replaced by either ATPγS or AMP-PNP (Fig. 5*B*). Significantly, the time course revealed a cycle of rapid formation and disruption of D-loop, such that the D-loop level reached its maximum by 1 min but declined rapidly thereafter (Fig. 5, *B* and *D*). In fact, by the reaction end point of 6 min, little or no D-loop remained (Fig. 5, *B* and *D*). Such a cycle of D-loop synthesis and reversal seems to be a general characteristic for the RecA/Rad51 class of recombinases (26, 27). Because the RecA-ssDNA nucleoprotein filament disassembles upon ATP hydrolysis (24), we considered the possibility that the dissociation of D-loop seen here (Fig. 5*B*) could be related to ATP hydrolysis-mediated turnover of hRad51. To test this premise, we used the hrad51 K133R mutant protein, which binds but does not hydrolyze ATP (20), with hRad54 in the D-loop assay. True to prediction, with hrad51 K133R, the D-loop amount increased with time, reaching a much higher final level than when hRad51 was used (Fig. 5, *B–D*); by 4 min, 23% of the input ssDNA or 55% of the pBluescript plasmid DNA had been incorporated into the D-loop structure. As expected, with both hRad51/hRad54 and hrad51 K133R/hRad54, formation of D-loop required both the 90-mer substrate and the pBluescript target, and substitution of the pBluescript DNA with the heterologous φX174 DNA completely abolished D-loop formation (data not shown).

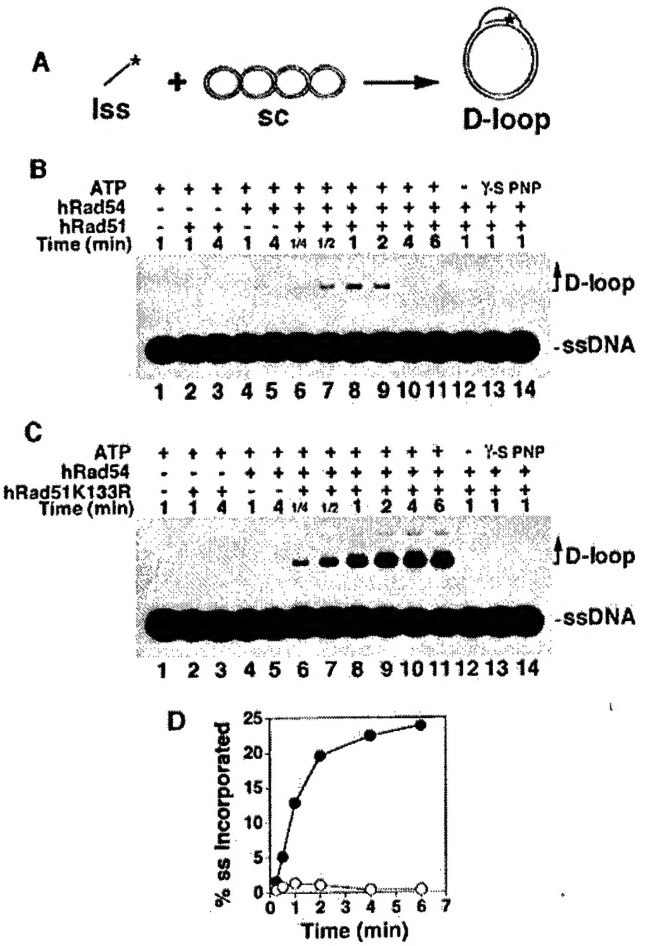

FIG. 5. **D-Loop formation by hRad51 and hRad54.** *A*, schematic of assay. A radiolabeled 90-mer DNA pairs with a homologous duplex target to yield a D-loop. *B*, hRad51 alone (*lanes 2* and *3*), hRad54 alone (*lanes 4* and *5*), and the combination of hRad51 and hRad54 (*lanes 6–14*) were incubated at 30 °C for the indicated times with the DNA substrates in the presence of ATP (*lanes 2–11*), ATPγS (γS; *lane 13*), or AMP-PNP (*PNP*; *lane 14*) or in the absence of nucleotide (*lane 12*). In *lane 1*, the DNA substrates were incubated in buffer without protein. The protein and DNA concentrations were as follows: hRad51, 800 nM; hRad54, 120 nM; 90-mer oligonucleotide, 2.5 μM nucleotides or 27.7 nM oligonucleotide; pBluescript supercoiled DNA, 35 μM base pairs or 11.6 nM plasmid. *C*, the homologous pairing activity of hrad51 K133R was examined with hRad54 as described for hRad51 above. *D*, the results in *lanes 6–11* of *B* (○) and *C* (●) were graphed.

DISCUSSION

It has been deduced from biochemical and scanning force microscopy analyses that Rad54 tracks on DNA, producing positive supercoils ahead of the protein movement and negative supercoils tailing it (15, 18). As a result of interaction with hRad51, the ATPase, DNA supercoiling, and DNA strand opening activities of Rad54 are greatly enhanced (this work). Petukhova *et al.* (19) first reported that yRad54 enhances homologous DNA pairing by yRad51. Here we have presented biochemical evidence that hRad51 and hRad54 also work in concert to make DNA joints. Interestingly, the hRad51/hRad54-mediated D-loop reaction undergoes a rapid cycle of joint formation and dissociation. We have speculated that ATP hydrolysis by hRad51 could have resulted in its turnover from the bound ssDNA. This might have led to the transfer of hRad51/hRad54 to the displaced strand in the D-loop to initiate a second round of homologous pairing with the newly formed DNA joint. The presumed secondary pairing reaction could have accounted for the dissociation of the initial D-loop. Consistent with this hypothesis, the ATP hydrolysis-defective hrad51 K133R mutant is much more adept at forming D-loop than the wild type protein. Previously, studies in yeast and chicken DT40 cells with the same Rad51 ATPase mutant have

shown that it is biologically active but that an increased level of this mutant is needed for full complementation of the various phenotypes of Rad51-deficient cells (20, 28). The fact that the hrad51 K133R mutant is even more effective than the wild type protein in the D-loop reaction strongly suggests that the slighted biological efficacy (20, 28) and observed dominance (29) of this protein are because of a reason other than a diminished ability to mediate homologous pairing. The hrad51 K133R mutant may form a highly stable complex with DNA, thus reducing the effective concentration of free protein available for recombination reactions. Importantly, our biochemical results predict that other members of the *RAD52* group may function to prevent reversal of the D-loop reaction catalyzed by hRad51/hRad54.

Inactivation of the hRad54 ATPase activity impairs the ability to carry out recombination *in vivo* (14), consistent with the premise that the ATP hydrolysis-dependent DNA supercoiling and DNA strand opening activities of hRad54 are germane for recombination. As discussed here and elsewhere (15, 18, 30), it is likely that the DNA strand opening activity of hRad54 promotes the acquisition of an unwound DNA structure conducive for the formation of the nascent DNA joint that links recombining chromosomes. The ability of hRad54 to pull the incoming duplex molecule through its fold (*i.e.* tracking) is also expected to enhance the rate at which the duplex can be sampled by the hRad51-ssDNA nucleoprotein filament for homology. Finally, it remains a distinct possibility that the dynamic DNA topological changes induced by the combination of hRad51 and hRad54 are critical for the remodeling of chromatin during recombination.

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